08-1606

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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

In re Application of:

Rea et al.

Serial No.: 09/666,430

Filed: September 21, 2000

For: DENDRITIC CELL ACTIVATED IN THE PRESENCE OF GLUCOCORTICOID HORMONES ARE CAPABLE OF SUPPRESSING ANTIGEN-SPECIFIC T CELL RESPONSES

Confirmation No.: 6289

Examiner: G. Ewoldt, Ph.D.

Group Art Unit: 1644

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BRIEF ON APPEAL

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Commissioner for Patents
P.O. Box 1450
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Sirs:

This brief is submitted as a single copy pursuant to 37 C.F.R. § 41.37 and in the format required by 37 C.F.R. § 41.37(c) (1):

(1) REAL PARTY IN INTEREST

The real party in interest in the present pending appeal is Leids Universitair Medisch Centrum (University of Leiden Medical Center), assignee of the pending application as recorded with the United States Patent and Trademark Office on October, 2000, at Reel 011197, Frame 0136.

(2) RELATED APPEALS AND INTERFERENCES

Neither the Appellants, the Appellants' representative, nor the Assignee is aware of any pending appeal or interference which would directly affect, be directly affected by, or have any bearing on the Board's decision in the present pending appeal.

(3) STATUS OF CLAIMS

Claims 2 through 39 were cancelled without prejudice or disclaimer

Claims 1 and 40 through 81 stand rejected.

No claims are allowed.

The rejections of claims 1 and 40 through 81 are being appealed.

(4) STATUS OF AMENDMENTS

The appellants' amendments, filed April 27, 2005 in conjunction with a Request for Continued Examination, have been entered.

(5) SUMMARY OF CLAIMED SUBJECT MATTER

The claimed invention provides means and methods for immunotherapy. The invention provides immune cells and methods to generate them, where the immune cells have the capacity, at least in part, to reduce an immune response in a host. *See*, Substitute Specification, mailed March 18, 2003, at page 6, lines 22-29. In one aspect, the invention provides a method for generating a dendritic cell with the capacity to tolerize a T-cell for the antigen the T-cell is specific for. *Id.*, at page 6 line 22 through page 7, line 12. More specifically, one aspect of the invention relates to culturing blood monocytes from a subject to differentiate into dendritic cells, activating the dendritic cells in the presence of a glucocorticoid hormone, and loading the

activated dendritic cell with an antigen that a T-cell is specific for. *See*, Specification at *Id.*, at page 6 line 22 through page 7, line 24; page 8, lines 1-9; page 11, lines 6-24.

As set forth in 37 C.F.R. 41.73 (c) (1) (vii), every means plus function claim must be identified and the structure materials or acts described in the specification corresponding to each claimed function must be set for the with reference to the specification. The current application contains a single means plus function claim, to wit: claim 1. The relevant means plus function language of claim 1 recites "activating said dendritic cells with a means for reducing IL-12p40 production by said dendritic cells."

The specification, in Example 3 (*See*, Substitute Specification, mailed March 18, 2003, at page 10, line 19 through page 11, line 2.), clearly indicates that dexamethasone, a known compound, has the ability to reduce reducing IL-12p40 production by a dendritic cell, and is thus a disclosed means to accomplish this function. Further, the Examiner, in the Office Action mailed July 26, 2005, at Page 5, agrees dexamethasone is a disclosed means for the function of reducing IL-12p40 production by said dendritic cells.

(6) GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

A. Whether claims 1, 46, and 55 are unpatentable under 35 U.S.C. § 112, first paragraph, as failing to comply with the written description requirement?

B. Whether claims 40-81 are unpatentable under 35 U.S.C. § 112, first paragraph, as constituting "new matter?"

C. Whether claims 1 and 40-81 are unpatentable under 35 U.S.C. § 112, first paragraph, as failing to require with the enablement requirement?

(7) ARGUMENT

(i) 35 U.S.C. § 112, first paragraph, written description

Claims 1, 46, and 55 stand rejected under 35 U.S.C. § 112, first paragraph, as assertedly failing to comply with the written description requirement.

As amended, independent claim 1 recites "a method for preparing a pharmaceutical composition for reducing an unwanted T-cell response in a host, said method comprising: culturing peripheral blood monocytes from said host to differentiate into dendritic cells; activating said dendritic cells with a means for reducing IL-12p40 production by said dendritic cells; loading said dendritic cells with an antigen against which said T-cell response is to be reduced; and forming a pharmaceutical composition comprising said loaded, activated dendritic cells for administration to said host."

As amended, dependent claim 46, including the element of independent claim 40, recites "a method for preparing a pharmaceutical composition for reducing an unwanted T-cell response in a host against an antigen, said method comprising: culturing peripheral blood monocytes from said host to differentiate into dendritic cells; activating said dendritic cells with a glucocorticoid capable of activating a glucocorticoid receptor; bringing said dendritic cells into contact with an antigen against which said T-cell response is to be reduced; and forming a pharmaceutical composition comprising said loaded, activated dendritic cells; wherein activating said dendritic cells with said substance capable of activating the glucocorticoid receptor comprises activating said dendritic cells such that said dendritic cells secrete interleukin-10."

As amended, dependent claim 55, including the elements of independent claim 51 and intervening claim 51, recites "a method for obtaining a dendritic cell capable of tolerizing a T-cell for an antigen, comprising: providing said dendritic cell with a substance capable of activating a glucocorticoid receptor; activating said dendritic cell; and providing said dendritic cell with said antigen, wherein said dendritic cell is capable of tolerizing a T-cell for said antigen; wherein providing said dendritic cell with the substance capable of activating a glucocorticoid receptor is in vitro; and wherein said substance capable of activating the glucocorticoid receptor enhances secretion of IL-10 by said dendritic cells."

Alleged Basis of the Rejections

As to claims 1, 46, and 55 the Examiner, in the Office Action mailed May 21, 2003 at Page 4, alleges that

> There is insufficient written description to show that Applicant was in possession of "means for reducing IL-12p40 production by said dendritic cell" or "means for

> causing said dendritic cell to secret IL-10 in vitro," other than dexamethasone. As said "means" comprises an unknown genus of indeterminate size, one of skill in the art must conclude that the specification fails to disclose an adequate written description or a representative number of species to describe the claimed genus. Likewise the specification discloses no specific "antigen(s) against which said T-cell response is to be reduced." Again, given the indeterminate size of the claimed "antigen" genus . . . one of skill in the art must conclude that the specification fails to disclose an adequate written description or a representative number of species to describe the claimed genus.

In addition, the Examiner alleges that "the singe substance capable of activating a glucocorticoid receptor disclosed in the speciation, *i.e.*, Dex, is not considered to be a representative number of examples of the claimed genus of all substances capable of activating a glucocorticoid receptor." Office Action mailed December 27, 2004 at Page 6.

Further, the Examiner asserts that "a representative number of examples is required." *Id.* The Examiner further alleges that "a single example is not a representative number of examples of the genus in this instance." *Id.*

Adequate Written Description For The Means Plus Function Element Of Claim 1 Exists In The Specification

35 U.S.C. § 112, Paragraph 6 relates that

> An element in a claim for a combination may be expressed as a means or step for performing a specified function without the recital of structure, material, or acts in support thereof, and such claims shall be construed to cover the corresponding structure, material, or acts described in the specification and equivalents thereof.

"The USPTO must apply 35 U.S.C. 112, sixth paragraph in appropriate cases, and give claims their broadest reasonable interpretation, in light of and consistent with the written description of the invention in the application. *In re Donaldson Co.*, 16 F.3d 1189, 29 USPQ2d 1845 (Fed. Cir. 1994). Under MPEP §2181(I), a claim element will be interpreted to invoke 35 U.S.C. 112, sixth paragraph, if it meets the following 3-prong analysis:

(A) the claim limitation must use the phrase "means for" or "step for;"

(B) the "means for" or "step for" must be modified by functional language; and

(C) the phrase "means for" or "step for" must not be modified by sufficient structure, material, or acts for achieving the specified function.

Claim 1 is the only claim at issue that contains a means plus function element. Specifically, claim 1 recites, in part, "activating said dendritic cells with a means for reducing IL-12p40 production by said dendritic cells." Prong (A) of the 3-prong test is clearly met as the claim clearly recites "means for." Prong (B) of the 3-prong test is also met as the means for is modified by function language, to wit: "means for reducing IL-12p40 production by said dendritic cells." Lastly, prong (C) of the 3-prong test is met as the "means for" recited in claim 1 is not modified by any structure, material, or acts for achieving the specified function. As such, claim 1 invokes clearly invokes 35 U.S.C. 112, sixth paragraph.

In rejecting claim 1, the Examiner alleges, that "as said 'means' comprises an unknown genus of indeterminate size, one of skill in the art must conclude that the specification fails to disclose an adequate written description or a representative number of species to describe the claimed genus." Office Action mailed 21, 2003 at Page 4. However, the Examiner's assertion regarding the size of the genus is in direct contravention to 35 U.S.C. § 112, sixth paragraph. As noted above, 35 U.S.C. § 112, sixth paragraph provides, in part, that "such claims shall be construed to cover the corresponding structure, material, or acts described in the specification and equivalents thereof." As such, the "means" recited in claim 1 cannot be construed as being of indeterminate size as the means must be construed to cover the corresponding structure, material, or acts described in the specification and equivalents thereof.

Further the MPEP at § 2163 (II)(3)(a) indicates that "A means- (or step) plus-function claim is adequately described under 35 U.S.C. § 112, paragraph 1, if the written description adequately links or associates adequately described particular structure, material or acts to the function recited in a means- (or step) plus function limitation."

The Examiner acknowledges that dexamethasone (dex) is a disclosed means for reducing IL-12p40 production by dendritic cells. Office Action mailed July 26, 2005 at Page 5. In so noting, the Examiner asserts that "it remains the Examiner's position that Dex is not a sufficiently representative number of means for reducing IL-12p40 production by a dendritic cell." *Id.* However, appellants are not required to provide an adequate number of species to define the genus. As the MPEP and the Federal Circuit have pointed out, written description is

satisfied if the specification adequately links a particular material to the function recited. *See In Re Donaldson, supra.* The Examiner, as noted above, agrees that Dex is a particular material that has been adequately linked to the function recited. Further, the specification, in Example 3 (¶ 34), indicates that dexamethasone, a known compound, has the ability to reduce reducing IL-12p40 production by a dendritic cell. As such, appellants respectfully submit that the specification provides adequate written description of a means for reducing IL-12p40 production by a dendritic cell and that the "means" recited in claim 1 does not comprise an unknown genus of indeterminate size. Consequently, appellants respectfully request that the rejection of claims 1 under 35 U.S.C., first paragraph, for lack of written description for recitation of the term "means" be withdrawn and the claim allowed.

The Term "Antigen" Does Not Give Rise To A Genus of Indeterminate Size In Claims 1, 46, and 55

To satisfy the written description requirement, a patent specification must describe the claimed invention in sufficient detail that one skilled in the art can reasonably conclude that the inventor had possession of the claimed invention. *See, e.g., Moba, B.V. v. Diamond Automation, Inc.*, 325 F.3d 1306, 1319, 66 USPQ2d 1429, 1438 (Fed. Cir. 2003). An applicant shows possession of the claimed invention by describing the claimed invention with all of its limitations using such descriptive means as words, structures, figures, diagrams, and formulas that fully set forth the claimed invention. *Lockwood v. American Airlines, Inc.,* 107 F.3d 1565, 1572, 41 USPQ2d 1961, 1966 (Fed. Cir. 1997). Possession may be shown in a variety of ways including description of an actual reduction to practice, or by showing that the invention was "ready for patenting" such as by the disclosure of drawings or structural chemical formulas that show that the invention was complete, or by describing distinguishing identifying characteristics sufficient to show that the applicant was in possession of the claimed invention. *See, e.g., Pfaff v. Wells Elecs., Inc.*, 525 U.S. 55, 68, 119 S.Ct. 304, 312, 48 USPQ2d 1641, 1647 (1998). Further, there is a strong presumption that an adequate written description of the claimed invention is present when the application is filed. *In re Wertheim*, 541 F.2d 257, 263, 191 USPQ 90, 97 (CCPA 1976).

The Examiner alleges, at page 6 of the Office Action mailed December 27, 2004, that "a representative number of examples is required" to establish written description. And that "[s]aid representative number can only be established in relation to the genus in question. *Id.* Appellants respectfully submit that the Examiner's requirement for a representative number of examples is contrary to established examination procedure. MPEP § 2163(II) (3) (a) (ii) provides that:

> The written description requirement for a claimed genus may be satisfied through sufficient description of a representative number of species by actual reduction to practice, reduction to drawings, or by disclosure of relevant identifying characteristics, *i.e.*, structure or other physical and /or chemical properties, by functional characteristics coupled with a known or disclosed correlation between function or structure, or by a combination of such identifying characteristics, sufficient to show the applicant was in possession of the claim genus. *See Regents of the University of California v. Eli Lilly*, 199 F.3d 1559, 1568, 43 USPQ2d 1398, 1406 (Fed. Cir. 1997).

As such, appellants respectfully assert that Examiner is mistaken in his assertion that "a representative number of examples is required," as established case law and the MPEP provide numerous alternatives to providing a representative number of species.

The Examiner further alleges that "the specification discloses no specific 'antigen(s) against which said T-cell response is to be reduced.'" Office Action mailed May 21, 2003 at Page 4. Appellants respectfully submit that which antigen a T-cell response is to be reduced is subjective in nature. The selection of the antigen is a decision of the practitioner of the invention and any antigen can be selected for use in practicing the present invention. Further, the appellants provide two examples of antigens to which were tested to see if a T-cell response to them was reduced. Specifically, Example 4 of the specification (¶ 35) shows the reduction of a T-cell response to the antigens hsp65 protein and p3-13. As such, given that the appellants selected hsp65 and p3-13 as antigens to which a T-cell response was to be reduced, and further demonstrated a reduction in T-cell response to these two antigens, appellants respectfully submit that the specification does disclose at least two examples of antigens to which a T-cell response is to be reduced.

To the extent that the Examiner is alleging that the appellants do not have possession of the genus defined by the term "antigen," appellants respectfully submit that one of skill in the art would conclude that appellants had possession of "antigens" as the genus encompassed by the term is well known in the art, examples of antigens are well known in the art, and exemplary "antigens" are reduced to practice in the examples as well as in the Declaration of Rienk Offiringa.

"Claims drawn to the use of known chemical compounds in a manner auxiliary to the invention must have a corresponding written description only so specific as to lead one having ordinary skill in the art to that class of compounds." *In re Herschler*, 591 F.2d 693, 702, 200 USPQ 711, 714 (CCPA 1979). In decision of the CCPA in *In re Herschler*, it was found that a single example of a steroid was sufficient to provide written description for the entire genus of steroids as the "[a]ppellant's invention is the combination claimed and not the discovery that certain inorganic salts have colloid suspending properties." *Id* at 701. Further the court noted that "[w]e see nothing in patent law which requires appellant to discover which of all those salts have such properties and will function properly in his combination." *Id.*

Appellants respectfully submit that the instant case sits on all four corners of the *In re Herschler* decision. In the present case, the applicant is claiming a specific combination of method steps in claims 1, 46, and 55 of which the selection of an antigen is only auxiliary to the claimed methods. As in *In re Herschler*, the invention defined by the present claims is not that specific antigens have a particular property or are novel in any way. The novelty of the claimed invention resides in the combination of all of the method steps.

Further, as in *In re Herschler*, antigens are known chemical compounds. Antigen is a term that is well defined in the art. The courts have recognized the definition of an antigen as a "foreign molecule of sufficient size can act as a stimulus for antibody production." Hybritech Inc. v. Monoclonal Antibodies, Inc. 802 F.2d 1367, 1368 (Fed. Cir. 1986). As such, the term "antigen" is well known in the art. Further, at least hundreds of antigens are known in the art. The following is a truncated exemplary list of know antigens: influenza viruses, immunoglobulin E (IgE) which indicates allergic reaction, human chorionic gonadotropin (HCG) which indicates pregnancy, and prostatic acid phosphatase (PAP) which indicates prostate cancer. *Id.* at 1370.

In addition, the specification and Declaration of Rienk Offringa, which is of record in the present case, provides examples of antigens that may be used in accordance with the present invention. Specifically, Example 2 of the specification (¶ 33) indicates the uptake of the antigens FITC-BSA and FITC-mannosylated BSA. Further, Example 4 of the specification (¶ 35) shows the use of the hsp65 protein and the specific peptide epitope p3-13 as antigens Last, the Declaration of Rienk Offringa, at page 9, indicates the use of C57BL/6 alloantigens and conA as antigens. As such, the specification and the Declaration of Rienk Offrenga show the use of no less than six antigens.

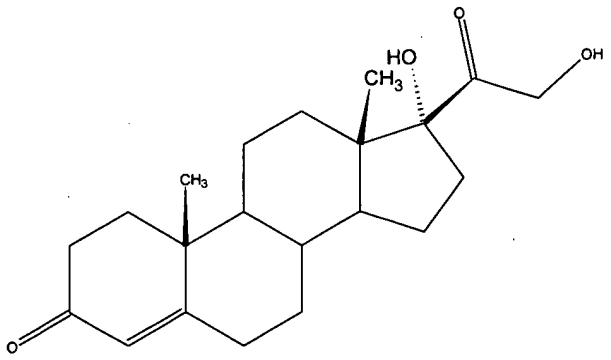
As directed by *In re Herschler*, where claims are drawn to the use of known compounds in a manner auxiliary to the invention, as is the case in the present application, the corresponding written description needs be only so specific as to lead one of ordinary skill in the art to that class of compounds. Appellants respectfully submit, as the term antigen is directly used in the claims, antigens are well known and characterized in the art, and as the specification and Declaration of Rienk Offrenga provide examples of antigens, one of skill in the art would be lead to use that class of compounds. As such, given direction of *In re Herschler*, appellants respectfully submit that the phrase "antigen(s) against which said T-cell response is to be reduced" and specifically the term "antigen" have adequate written description in the specification. Consequently, appellants respectfully request that the rejection of claims 1, 46, and 55 under 35 U.S.C., first paragraph, for lack of written description be withdrawn and the claims allowed.

Substances Capable Of Activating A Glucocorticoid Receptor Recited in Claims 46, and 55 Are Well Known In The Art

In rejecting claims 46 and 55 the Examiner alleges that:

> The single substance capable of activation a glucocorticoid receptor disclosed in the specification, *i.e.*, Dex, is not considered to be a representative number of examples of the claimed genus of all substances capable of activating a glucocorticoid receptor. Applicant is reminded, as set forth previously, that the term must be given its broadest reasonable interpretation, such as set for the in *Stedman's Medical Dictionary* (2002) wherein glucocorticoid is defined as "any steroid-like compound capable of significantly influencing intermediary metabolism."

As noted *supra*, a representative number of examples of the claimed genus is not required. *Eli Lilly* at 1559. An additional option for compliance with the written can be provided by disclosure of relevant identifying characteristics, *i.e.*, structure or other physical and/or chemical properties. *Id.* As is well known in the art, glucocorticoids (*e.g.* chemicals capable of activating the glucocorticoid receptor) have a common base chemical structure; to wit:



Goodman *et al.*, Goodman and Gilman's The Pharmacological Basis of Therapeutics, (Seventh Ed.) Ch 63 Adrenocorticotrophic Hormone: Adrenocortical Steroids and their Synthetic Analogs: Inhibitors of Andrenocortical Steroid Biosynthesis, page 1464. Further, the use of glucocorticoids in the present invention is discussed in each of ¶¶ 18-20, As such, appellants respectfully submit that adequate written description of substances capable of activating a glucocorticoid receptor exists as the use of glucocorticoids is discussed in multiple locations in the specification and glucocorticoids are a well known class of compounds with common chemical properties. Consequently, appellants respectfully request that the rejection of claims 46, and 55 under 35 U.S.C., first paragraph, for lack of written description be withdrawn and the claims allowed.

In addition, as noted *supra*, claims drawn to the use of known chemical compounds in a manner auxiliary to the invention must have a corresponding written description only so specific as to lead one having ordinary skill in the art to that class of compounds." *In re Herschler*, 591 F.2d at 702. In decision of the CCPA in *In re Herschler*, it was found that a single example of a steroid was sufficient to provide written description for the entire genus of steroids as the '[a]ppellant's invention is the combination claimed and not the discovery that certain inorganic salts have colloid suspending properties." *Id* at 701. Further the court noted that "[w]e see

nothing in patent law which requires appellant to discover which of all those salts have such properties and will function properly in his combination." *Id.*

Appellants respectfully submit that the instant case sits on all four corners of the *In re Herschler* decision. In the present case, the applicant is claiming a specific combination of method steps in claims 46, and 55 of which the use of a specific substance capable of activating a glucocorticoid receptor is only auxiliary to the claim methods. As in *In re Herschler*, the invention defined by the present claims is not that specific glucocorticoids are novel in any way, the novelty of the claimed invention resides in the combination of all of the method steps.

Further, as in *In re Herschler*, compositions capable of activating a glucocorticoid receptor are well known chemical compounds. Goodman and Gilamn defines a glucocorticoid as 21 carbon steroids with the general structure illustrated *supra*. Goodman *et al.*, at page 1464. As such, substances capable of activating the glucocorticoid receptor (*i.e.* glucocorticoids) are well known in the art. Further, multiple examples of glucocorticoids are known in the art. The following is a truncated exemplary list of know glucocorticoids: betamethasone, cortisone, dexamethasone, fluldrocortisone, methylprednisolone, paramethasone, prednisolone, prednisone, and triacinolone. *Id.* at page 1475. Only minor differences distinguish these glucocorticoids including the presence of a double bond at the 1-2 position, and the presence or absence of various small auxiliary groups at positions 6, 9, 11, and 16. *Id.* at pate 1474.

In addition, the specification provides an example of a glucocorticoid (dexamethasone) that may be used in accordance with the present invention to activate a glucocorticoid receptor. Specifically, Examples 1-4 of the specification disclose use of dexamethasone a dosage 1000 times the level needed to fully activate the glucocorticoid receptor (*see* ¶ 38 of the specification and Cronstein *et al.* (1992) Proc. Natl. Acad. Sci. USA 89:9991-9995 at Figures 1 and 3.)

As directed by *In re Herschler*, where claims are drawn to the use of known compounds in a manner auxiliary to the invention, as is the case in the present application, the corresponding written description needs be only so specific as to lead one of ordinary skill in the art to that class of compounds. Appellants respectfully submit, as the use of a substance capable of activating a glucocorticoid receptor is disclosed in the specification, substance capable of activating a glucocorticoid receptor (*i.e.* glucocorticoids) are well known and characterized in the art, and as the specification provides a specific example and use of such a substance, one of skill in the art

would be lead to use that class of compounds. As such, given direction of *In re Herschler*, appellants respectfully submit that the phrase "substance capable of activating a glucocorticoid receptor" has adequate written description in the specification. Consequently, appellants respectfully request that the rejection of claims 46, and 55 under 35 U.S.C., first paragraph, for lack of written description be withdrawn and the claims allowed.

(ii) 35 U.S.C. § 112, first paragraph, written description, new matter

Claims 40-81 stand rejected under 35 U.S.C. § 112, first paragraph, as allegedly constituting new matter.

Specifically, the Examiner alleges, in the Office Action mailed December 27, 2004, at page 7, that:

> The specification and the claims as originally filed do not provide support of the invention as now claimed, specifically:
> A) A substance capable of activating a glucocorticoid receptor (claims 40 and 51-55)
> B) A method for preparing an isolated dendritic cell, said method comprising:
> isolating peripheral blood monocytes from a subject;
> culturing the peripheral blood monocytes to differentiate into dendritic cells;
> activating the dendritic cells with a glucocorticoid;
> loading the dendritic cells with and antigen; and
> isolating said loaded, activated dendritic cells (claim 56)
> C) The method according to claim 56, wherein the antigen comprises an allogenic antigen (claim 59)
> D) A method for obtaining a dendritic cell capable of tolerizing a T-cell in a graft or transplant recipient (claims 64 and 65).
>
> Regarding A) the specification supports binding but not activating.
> Regarding B) the specification does not disclose this generic method for preparing any type of isolated DC.
> Regarding C) the specification does not disclose the generically-claimed method employing a generic allogenic antigen.
> Regarding D) the specification discloses only tolerizing T cells to an antigen and not tolerzing a generic T-cell or tolerzing a T-cell in a graft or transplant recipient.

The Examiner further alleges, in the Office Action mailed July 26, 2006, at pages 7-9, that the following new claims also constitute new matter, noting that:

A) A method for preparing a pharmaceutical composition for reducing an unwanted T-cell response to an antigen in a host, said method comprising:

culturing peripheral blood monocytes form said host to differentiation into dendritic cells *in vitro*;

contacting said dendritic cells *in vitro* with an antigen against which said T-cell response is to be reduced, thereby loading said dendritic cells with the antigen;

contacting said dendritic cells with dexamethasone;

activating the CD40 receptor on said dendritic cells; and

forming a pharmaceutical composition comprising said loaded, activated dendritic cells (claim 69).

(B) The method of claim 69 comprising the additional limitation of claims 70-76.

(C) A method for obtaining a dendritic cell capable of tolerizing a T-cell for an antigen, the method comprising:

contacting a dendritic cell with dexamethasone *in vitro*;

activating the dendritic cell through the CD40 receptor; and

contacting the dendritic cell with an antigen, thereby loading the dendritic cell with the antigen, and forming a dendritic cell capable of tolerzing a T-cell for the antigen (claim 77).

(D) The method of claim 77 comprising the additional limitations of claims 78-81.

(E) The methods of claims 43 and 75 comprising incubating DCs with a peptide antigen.

(F) The method of claim 53 comprising providing a precursor of a DC.

(G) The method of claim 58 comprising loading DCs with an antigen defined by the response of a T cell.

(H) The method of claims 61, 65, 68, 78, 80, and 81 comprising DCs derived from a graft or transplant donor.

A review of the specification shows no support for the specific order of the steps in claim 69, *e.g.*, contacting the cells with antigen before contacting the DCs with Dex before activating the DCs. Nor does the specification support the specific order of the steps in claim 77, *e.g.*, contacting the cells with Dex before activating the DCs before contacting the DCs with antigen.

Regarding (E), the specification does not disclose peptide antigens and original claim 9 discloses only synthetic peptides.

Regarding (F), the specification does not disclose "a precursor of a DC."

Regarding (G), the specification does not disclose "an antigen defined by the response of the T cell."

Regarding (H), the specification does not disclose "DCs derived from a graft or transplant donor."

To comply with the written description require of 35 U.S.C. § 112, first paragraph, each claim limitation must be expressly, implicitly, or inherently support in the originally filed disclosures. MPEP § 2163(II)(A)(3)(b). Further, the subject matter of the claim need not be described literally, *in haec verba*, in order for the disclosure to comply with the written description requirement. MPEP § 2163.02.

As To Claim 40 And 51-55, Activating A Glucocorticoid Has Support In The Specification

As noted *supra*, compliance with the written description requirement can be met if a claim limitation is implicitly or inherently supported in the originally filed disclosures. MPEP § 2163(II)(A)(3)(b). The Examiner indicates, in the Office Action mailed December 27, 2004, at page 7, that "the specification supports binding [of Dex to a glucocorticoid receptor] but not activating [a glucocorticoid receptor with Dex]." However, appellants respectfully submit that the agreed upon binding of Dex to the glucocorticoid receptor implicitly and inherently results in its activation. That Dex activates the glucocorticoid receptor is well known in the art. For example, Cronstein *et al.* indicate that "antagonism by dexamethasone . . . is a specific instance of the general biological principle that the glucocorticoid receptor is a hormone dependent regulator of transcription. Cronstien B.M., Kimmel S.C, Levin R.I., Martinuik F., and Weismann G: A mechanism for the anti-inflammatory effect of corticosteroids: the glucocorticoid receptor regulates leukocyte adhesion to endothelial cells and expression of endothelial-leukocyte adhesion molecule 1 and intercellular adhesion molecule 1. (1992) Proc. Natl. Acad. Sci. USA 89:9991-9995 at the abstract. Further, the experiments of Cronstein *et al.* reached a maximal effect on the glucocorticoid receptor at 10nM dexamethasone. *Id.* at Figures 1 and 3. Appellants, as noted in ¶ 38 of the specification, use 10mM dexamethasone, 1000 times the level indicated by Cronstein *et al.* to achieve maximal activation of the glucocorticoid receptor. Last, the Examiner acknowledges that dexamethasone is capable of activating a glucocorticoid receptor, to wit: "[a]pplicant is advised that the single substance capable of activating receptor disclosed in the specification, *i.e.*, dex . . . ." Office Action mailed December 27, 2004, at Page 6. As such, given the known ability of dexamethasone to activate the

glucocorticoid receptor, the amount of dexamethasone used by the appellants in their examples to activate the glucocorticoid receptor, and the statement by the examiner that dexamethasone is capable of activating the glucocorticoid receptor, one of skill in the art would conclude that the specification at the very least implicitly and inherently provides written description for the activation of the glucocorticoid receptor. Consequently, appellants respectfully request that the rejection of claims 40, and 51-55 under 35 U.S.C., first paragraph, as constituting new matter be withdrawn and the claims allowed.

The Specification Discloses The Method of Claim 56

As noted *supra*, claim 56 recites:

A method for preparing an isolated dendritic cell, said method comprising:
isolating peripheral blood monocytes from a subject;
culturing the peripheral blood monocytes to differentiate into dendritic cells;
activating the dendritic cells with a glucocorticoid;
loading the dendritic cells with and antigen; and
isolating said loaded, activated dendritic cells.

Appellant respectfully submit that support for claim 56 can be found throughout the specification. Specifically, original claim 1 provides support for many of the steps recited in claim 56. Original claim 1 recites:

A method for preparing a pharmaceutical composition for reducing an unwanted Tcell response in a host, comprising

culturing peripheral blood monocytes from said host to differentiate into dendritic cells,

activating said dendritic cells in the presence of a glucocorticoid hormone and

loading said dendritic cells with an antigen against which said Tcell response is to be reduced.

The step of "culturing peripheral blood monocytes from said host to differentiate into dendritic cells" in original claim 1 inherently and implicitly provides written description of the steps of "isolating peripheral blood monocytes from a subject" and "culturing the peripheral blood monocytes to differentiate into dendritic cells" that are recited in claim 56. The step of claim 1 and the steps of claim 56 amount to the same act as they give the same result using the same steps but are laid out in slightly different language. Further support for these steps of claim

56 are found in ¶ 36 of the specification. Specifically, that paragraph relates "[h]uman [peripheral blood monocytes precursors] from healthy donors, [were] isolated." Further ¶ 36 of the specification relates that "[i]mmature DC were generated from peripheral blood monocytes precursors." Thus, appellants respectfully submit that support for the claim 56 steps of "isolating peripheral blood monocytes from a subject" and "culturing the peripheral blood monocytes to differentiate into dendritic cells" are at least supported in original claim 1 and in ¶ 36 of the specification. Other support for these steps can be found throughout the specification.

The step of "activating the dendritic cells with a glucocorticoid" of claim 56 also has support throughout the specification. In particular, activating the heretofore unknown developmental pathway of dendritic cells with glucocorticoid is described in ¶¶ 18-22.

Further, the step of "loading the dendritic cells with an antigen" is supported throughout the specification. In particular, as noted *supra*, original claim 1 specifically recites "loading said dendritic cells with an antigen against which said T-cell response is to be reduced." As such, this particular method step is at least supported as the specific step is found in original claim 1.

Last, the step of "isolating said loaded, activated dendritic cells" is supported throughout the specification. In particular, ¶ 25 of the specification provides for "an isolated functionally modified T-cell obtainable by a method according to the invention. As such, appellants respectfully submit that the last step of claim 56 is explicitly described in at least ¶ 25 of the specification.

In view of the foregoing, appellants respectfully submit that each and every element of claim 56 is supported in the specification and originally filed claims. Consequently, appellants respectfully request that the rejection of claim 56 under 35 U.S.C., first paragraph, as constituting new matter be withdrawn and the claim allowed.

Support Exists In The Specification For Claim 59

As noted *supra*, claim 59 stands rejected as constituting new matter. Specifically, the Examiner, at Page 7 of the Office Action mailed December 27, 2004, notes that "[t]he cited paragraphs do not disclose the limitations of the claim, *e.g.*, the preparation of a generic isolated DC employing peripheral blood monocytes and an allogenieic antigen. Note that paragraph 35

does disclose an alloantigen, but the disclosure is made in a specific example and cannot support a generic method."

Claim 59 recites "[t]he method according to claim 56, wherein the antigen comprises an allogenic antigen."

By disclosing in a patent application a device that inherently performs a function or has a property, operates according to a theory or has an advantage, a patent application necessarily discloses that function, theory or advantage, even though it says nothing explicit concerning it. MPEP § 2163.07(a); *In re Reynolds*, 443 F.2d 384, 170 USPQ 94 (CCPA 1971). The application may later be amended to recite the function, theory or advantage without introducing prohibited new matter. *Id.*

As noted *supra*, support exists in the specification for the term "antigen." As would be understood by one of skill in the art, "antigen" is a broad term that encompasses generally many different subtypes of antigens. Further, appellants are entitled to the broadest reasonable interpretation of the language. *See e.g.*, *In re Morris*, 127 F.3d 1048, 1053-54, 44 USPQ2d 1023, 1027 (Fed. Cir. 1997). As would be understood by one of skill in the art, given the broadest reasonable interpretation of the term "antigen," the use of antigens as described in the specification inherently has the properties all subtypes of antigens. As such, appellants respectfully submit that adequate written description for the term "allogenic antigen" exists in the specification.

Further, the courts have defined allogenic as "[i]ntraspecies variance at a particular gene locus. Referring to genetic variants within a species." *Johns Hopkins University v. CellPro*, 894 F.Supp. 819, 825 (D.Del.,1995). As is well known in the art, antigenically distinct grafts or transplants may give rise, through allogenic antigens, to an unwanted immune response that characterizes one aspect of graft-versus-host or host-versus-graft disease. The specification, at ¶¶ 7, 18, and 29 specifically note the use of the invention in treating these diseases. Thus, one of skill in the art would understand that in order to combat these diseases as disclosed in the specification, the antigens described in the specification must inherently include allogenic antigens.

As such, appellants respectfully submit that the term "antigen," given its broadest reasonable interpretation inherently describes allogenic antigens, and that one of skill in the

would understand that allogenic antigens are inherently disclosed in the specification as antigens usefully in the disclosed methods for treating graft-versus-host or host-versus-graft disease. Consequently, appellants respectfully request that the rejection of claim 59 under 35 U.S.C., first paragraph, as constituting new matter be withdrawn and the claims allowed.

Claims 64 And 65 Specifically Recite Tolerizing A T-Cell To An Antigen

As noted *supra*, claims 64 and 65 stand rejected as constituting new matter. Specifically, the Examiner, at Page 7 of the Office Action mailed December 27, 2004, alleges that "[t]he specification discloses only tolerizing T cells to an antigen and not tolerizing a generic T-cell or tolerizing a T-cell in a graft or transplant recipient."

Contrary to the Examiner's above assertion, claims 64 and 65 do recite tolerizing T-Cells to an antigen. Claim 64 recites, in the preamble, "[a] method for preparing a dendritic cell capable of tolerizing a T-cell, said method comprising." However, the preamble of claim 64 is further informed by the method step of "loading the dendritic cells with an antigen which is MHC-matched to a clonal T-cell, wherein the dendritic cells are capable of tolerizing the clonal T-cell in vitro to the antigen." As such, by its own recited method steps, claim 64 describes tolerizing a T-cell to an antigen, which the Examiner has agreed has support in the specification.

Claim 65 recites, in the preamble, "[a] method for preparing a dendritic cell for tolerizing a T-cell in a graft or transplant recipient, said method comprising." However, as in claim 64, the preamble of claim 65 is informed by the recited method step of "loading-said dendritic cells with an antigen against which said T-cell is to be tolerized." Again, by its own recited method steps, claim 65 describes tolerizing a T-cell to an antigen, which the Examiner has agreed has support in the specification.

To the extent it may be relevant that the preamble recites the use of the claimed method in treating a graft or transplant recipient, support for such a use is found through out the specification. Specifically, the specification, at ¶¶ 7, 18, and 29 specifically notes the use of the invention in treating graft-versus-host and host-versus-graft disease. These diseases only arise in graft or transplant recipients. As such, the specification provides support for tolerizing a T-cell in a graft or transplant recipient.

Given that the objected to new matter appears only in the preambles and that the preambles of claims 64 and 65 are informed by recited method steps indicating the tolerization of a T-cell to an antigen, appellants respectfully submit that the specification provides adequate written description for claims 64 and 65. Consequently, appellants respectfully request that the rejection of claims 64 and 65 under 35 U.S.C., first paragraph, as constituting new matter be withdrawn and the claims allowed.

The Order Of Steps In Claims 69 Through 81 Have Support In The Specification

As noted *supra*, the Examiner has rejected claims 69 through 81 as constituting new matter as there is allegedly "no support for the specific order of the steps in claim 69, *e.g.*, contacting the cells with antigen before contacting the DCs with Dex before activating the DCs. Nor does the specification support the specific order of the steps in claim 77, *e.g.*, contacting the cells with Dex before activating the DCs before contacting the DCs with antigen." Office Action mailed July 26, 2005, at Page 8.

Unless the steps of a method actually recite an order, the steps are not ordinarily construed to require one. However, such a result can ensue when the method steps implicitly require that they be performed in the order written. *Interactive Gift Exp. Inc. v. Compuserve Inc.*, 257 F.3d 1323, 59 USPQ2d 1401 (Fed. Cir. 2001). There is a two-part test for determining if the steps of a method claim that do not otherwise recite an order must nonetheless be performed in the order in which they are written. First, the court looks to the claim language to determine if, as a matter of logic or grammar, they must be performed in the order written. *Altiris Inc. v. Symantec Corp.*, 318 F.3d 1363, 65 USPQ2d 1865 (Fed. Cir. 2003). If not, it next looks to the rest of the specification to determine whether it directly or implicitly requires such a narrow construction. *Id.*

Claims 69 and 77, from which claims 70-76 and 78-81 depend respectively, recite the transitional phrase "said method comprising." As such, the preamble indicates that certain steps must be followed, but gives no logical or grammatical indication that the steps must be performed in a specific order. Further, the claim elements at issue do not grammatically refer to one another in such away so as to logically or grammatically indicate that they must be performed in the order written. The steps at issue of claim 69 recite:

contacting said dendritic cells *in vitro* with an antigen against which said T-cell response is to be reduced, thereby loading said dendritic cells with the antigen;
contacting said dendritic cells with dexamethasone;
activating the CD40 receptor on said dendritic cells.

None of these steps make any reference to one another in such a way to indicate logically or grammatically that anyone of the steps must take place before any other. Each step recites doing something to dendritic cells, and there is nothing to indicate in what order these steps have to be performed on the dendritic cells.

Similarly, the steps at issue in claim 77 recite:

contacting a dendritic cell with dexamethasone *in vitro*;
activating the dendritic cell through the CD40 receptor;
contacting the dendritic cell with an antigen, thereby loading the dendritic cell with the antigen.

As with claim 69, none of these steps make any reference to one another in such a way to indicate logically or grammatically that anyone of the steps must take place before any other. Each step recites doing something to dendritic cells, and there is nothing to indicate in what order these steps have to be performed on the dendritic cells.

As the there is nothing logically or grammatically present in the claims indicating in what order the steps at issue should be pursued, we should next look to the rest of the specification to determine whether it directly or implicitly requires such a narrow construction. *Altiris Inc. v. Symantec Corp., surpa.*

As to claim 69, support exists in the specification for contacting the cells with antigen before contacting the DCs with Dex before activating the DCs. Specifically, ¶ 35 recites testing T-cells exposed to "p3-13-pulsed DEX-treated CD40-triggered DC." This description of the DC follows exactly the listing of the elements at issue in claim 69. To wit: A) contacting cells with antigen relates to "p3-13 pulsed;" B) contacting the cells with Dex relates to "DEX-treated;" and C) activating the DCs relates to "CD40-triggered DC." Thus, appellants respectfully submit that a dendritic cell described using the precise order of steps outlined in claim 69 is recited in the specification.

As to claim 77, support exists in the originally filed claims for contacting the cells with

Dex before activating the DCs before contacting the DCs with antigen. To wit: originally filed claim 1 recites, in relevant part, "activating said dendritic cells in the presence of a glucocorticoid hormone; and loading said dendritic cells with an antigen against which said T-cell response is to be reduced." Further, original claim 13 recites:

> A method for obtaining a dendritic cell capable of tolerizing a T-cell for an antigen, comprising:
> providing said dendritic cell with a glucocorticoid hormone;
> activating said dendritic cell; and
> providing said dendritic cell with said antigen.

In comparison to claim 77, the same order of steps is recited. To wit: A) "providing said dendritic cell with a glucocorticoid hormone" relates to "contacting the cells with Dex;" B) "activating said dendritic cell" relates to "activating the DCs;" and C) "providing said dendritic cells with said antigen" relates to "contacting the DCs with an antigen." As such, appellants respectfully submit that originally filed claim 13 specifically recites the same order of steps present in claim 77.

As the language of the claims does not logically or grammatically require the steps at issue to be performed in any specific order, and as the order of the steps in claims 69 and 77 are supported in the specification and originally filed claims, appellants respectfully submit that claims 69 and 77 do not introduce new matter. Consequently, appellants respectfully request that the rejection of claims 69 and 77 under 35 U.S.C., first paragraph, as constituting new matter be withdrawn and the claims allowed. Further, as claims 70-76 and 78-81 depend from claims 69 and 77 respectively, appellants respectfully request that these claims are allowable at least as depending from an allowable independent claim.

The "Peptide Antigen" Of Claims 43 and 75 Has Support In The Specification

As noted *supra*, the Examiner has rejected claims 43 and 75 as constituting new matter as allegedly "[t]he specification does not disclose peptide antigens and original claim 9 discloses only synthetic peptides." Office Action mailed July 26, 2005, at Page 8.

Appellants respectfully submit that support for "peptide antigens" exists throughout the specification. Specifically, ¶ 19 of the specification indicates that "[a]n antigen is typically a

peptide capable of binding to a major histocompatibility complex (MHC) I and/or II molecule." Further, in the same paragraph, the specification goes on to relate that "[a]n antigen may also be a synthetic peptide," clearly indicating that the previous use of the term "peptide" was meant to include naturally occurring as well as synthetic peptides. In addition, Example 4 (¶ 35 of the specification) relates the use of hsp65 protein and the peptide epitope p3-13 as antigens. Both of these antigens constitute naturally occurring peptides. As such, appellants respectfully submit that support exists in the specification for the use of peptide antigens at least as the specification, reasonably read, indicates that both naturally occurring and synthetic peptide antigens may be part of the invention, and as one example of the specification uses naturally occurring peptides as antigens. Consequently, appellants respectfully request that the rejection of claims 43 and 75 under 35 U.S.C., first paragraph, as constituting new matter be withdrawn and the claims allowed.

The "Precursor Of A Dendritic Cell" Of Claim 53 Has Support In The Specification

As noted *supra*, the Examiner has rejected claim 53 as constituting new matter as allegedly "the specification does not disclose 'a precursor of a DC.'" Office Action mailed July 26, 2005, at Page 8.

Appellants respectfully submit that support for "a precursor of a dendritic cell" exists throughout the specification. Specifically, ¶ 18 of the specification indicates that "culturing peripheral blood monocytes from the host to differentiate into dendritic cells." As such, the specification, at ¶ 18, clearly indicates that the appellants were in possession of a precursor to dendritic cells, to wit: peripheral blood monocytes. Language identical to the above noted portion of ¶ 18 also appears at ¶¶ 19 and 20. In addition, ¶ 36 of the specification specifically outlines methodology that may be used to generate dendritic cells from their precursors, peripheral blood monocytes.

Furthermore, original claim 1 recites "culturing peripheral blood monocytes from said host to differentiate into dendritic cells." As with the cited portions of the specification, original claim 1 clearly indicates that the appellants were in possession of a precursor to dendritic cells.

Further, original claim 29 recites:

The method according to claim 13, wherein providing said dendritic cell with a

glucocorticoid hormone comprises providing a precursor of said dendritic cell with said glucocorticoid hormone in vitro.

As such, original claim 29 further indicates that the appellants were in possession of a precursor of a dendritic cell.

In light of the discussion *supra*, appellants respectfully submit support exists in the specification for "precursors of dendritic cell" at least as the specification, indicates that the appellants were in possession of a precursor of dendritic cells and methods to generate dendritic cells from this precursor. Further, original claim 29 clearly indicates that the appellants were *ipsis verbis* in possession of a precursor of a dendritic cell. Consequently, appellants respectfully request that the rejection of claim 53 under 35 U.S.C., first paragraph, as constituting new matter be withdrawn and the claim allowed.

The "Antigen Defined By The Response Of A T-Cell" Of Claim 58 Has Support In The Specification

As noted *supra*, the Examiner has rejected claim 58 as constituting new matter as allegedly "the specification does not disclose 'an antigen defined by the response of a T-cell.'" Office Action mailed July 26, 2005, at Page 8.

Appellants respectfully submit that support for an "antigen defined by the response of a T-cell" exists throughout the specification. Specifically, ¶ 22 of the specification notes that "the invention provides a method for obtaining a dendritic cell capable of tolerizing a T-cell for an antigen comprising . . . providing the dendritic cell with the antigen." As is well known in the art, T-cells have individually variable receptors, directly analogous to the antibodies, that give a particular T-cell a unique ability to detect particular antigens presented by antigen presenting cells. As a particular T-cell will only responds to particular antigens, it is implicitly and inherently described in ¶ 22 of the specification that once the T-cell response to be tolerized is identified, the proper antigen to tolerize it can be selected. Thus, implicit and inherent written description for "an antigen defined by the response of a T-cell" exists in the specification.

Further, ¶ 7 of the specification indicates that "the present invention, therefore, indicates that such DC loaded with appropriate antigens can be exploited as a novel approach for specifically down regulating unwanted T-cell responses in vivo." As above, this statement of the

specification provides implicit an inherent support for "an antigen defined by the response of a T-cell." Specifically, the appropriate antigens are selected based on the unwanted T-cell response. In this manner, the appropriate antigens are defined by the unwanted response of a T-cell.

As such, appellants respectfully submit that adequate written description exists in the specification for the phrase "an antigen defined by the response of a T-cell" as recited in claim 58. Consequently, appellants respectfully request that the rejection of claim 58 under 35 U.S.C., first paragraph, as constituting new matter be withdrawn and the claim allowed.

The "DCs Derived From A Graft Or Transplant Donor" Of Claims 61, 65, 68, 78, 80, And 81 Has Support In The Specification

As noted *supra*, the Examiner has rejected claims 61, 65, 68, 78, 80, and 81 as constituting new matter as allegedly "the specification does not disclose 'DCs derived from a graft or transplant donor.'" Office Action mailed July 26, 2005, at Page 9.

To comply with the written description require of 35 U.S.C. § 112, first paragraph, each claim limitation must be expressly, implicitly, or inherently support in the originally filed disclosures. MPEP § 2163(II)(A)(3)(b). Further, the subject matter of the claim need not be described literally, *in haec verba*, in order for the disclosure to comply with the written description requirement. MPEP § 2163.02.

Appellants respectfully submit that support for "DCs derived from a graft or transplant donor" exists throughout the specification. Specifically, ¶ 28 of the specification provides that

> The invention also provides a method for the treatment of an individual suffering from, or at risk of suffering from, a disease associated with at least part of the immune system of the individual , including providing the individual with a dendritic cell and/or a functionally modified T-cell . Preferably, the dendritic cell and/or the functionally modified T-cell or precursors thereof are derived from an HLA-matched donor. Preferably, the HLA-matched donor is the individual.

This paragraph of the specification implicitly and inherently provides that the dendritic cells may be obtained from a graft or transplant donor. The paragraph cited indicates that it is preferable that the dendritic cells to be modified come from the individual to be treated, but in so stating implicitly and inherently supports the notion that dendritic cells may come from other sources,

such are a donor of a graft or transplant. Further, the cited paragraph indicates that it is preferable that the dendritic cells are derived from an HLA-matched donor. As donors of transplants and grafts will very typically be HLA-matched to the recipient to aid in avoiding rejection, such a statement implicitly and inherently supports the notion that dendritic cells may come from the donor of a graft or transplant. Consequently, appellants respectfully request that the rejection of claims 61, 65, 68, 78, 80, and 81 under 35 U.S.C., first paragraph, as constituting new matter be withdrawn and the claims allowed.

(iii) 35 U.S.C. § 112, first paragraph, lack of enablement

Claims 1 and 40-81 stand rejected as allegedly failing to comply with the enablement requirement of 35 U.S.C. § 112, first paragraph.

The Examiner in the Office Action mailed Jun 29, 2001, at Page 4, asserts that

> The specification, while being enabling for the in vitro induction of non-responsiveness of MHC matched clonal T-cells to a defined antigen when dexamethasone-treated dendritic cells have been loaded with the same defined antigen does not reasonably provide enablement for in vivo or in vitro induction of non-responsiveness of polyclonal T cells to any undefined antigen or the in vivo induction of non-responsiveness when an "un-wanted T cell response" is ongoing.

In response to the appellants arguments, the Examiner further alleges, in the Office Action mailed September 19, 2002, at pages 2 and 3, that

> It appears then that the applicant is arguing that the invention of the instant claims function through a previously undescribed mechanism. Said mechanism would them be considered unexpected and accordingly, unpredictable. Given the unpredictability of the invention of the instant claims, some sort of enablement, in addition to mere assertion would be required.

In the Office Action mailed May 21, 2003, the Examiner, at page 3, indicates that:

> The rejection is based on two key factors. First the specification fails to disclose precisely how the antigens that induce unwanted T cell responses are established. Second, given that the claims are drawn to a method for preparing a pharmaceutical composition, the specification fails to adequately disclose that the DCs would function as a pharmaceutical composition *in vivo*.

The test of enablement is whether one reasonably skilled in the art could make or use the invention from the disclosures in the patent coupled with information known in the art without undue experimentation. *In re Buchner* 929 F.2d 660, 662, 18 USPQ2d 1331, 1332 (Fed. Cir. 1991). Further, a patent need not teach, and preferably omits, what is well known in the art. *Id.* To comply with the enablement requirement, it is not necessary to enable one of ordinary skill in the art to make a use a perfected commercially viable embodiment of absent a claim limitation to that effect. *CFMT, Inc. v. Yieldup Int'l Corp.*, 349 F.2d 1333, 1338, 68 USPQ2d 1940, 1044 (Fed. Cir. 2003). Detailed procedures for making and using the invention may not be necessary if the description of the invention itself is sufficient to permit those of skill in the art to make and use the invention. MPEP § 2164. All that is necessary is that one skilled in the art be able to practice the claimed invention, given the level of knowledge and skill in the art. *In re Fisher*, 4127 F.2d 833, 839, 166 USPQ 18, 24 (CCPA 1970).

Appellants respectfully submit that the specification, along with the Declaration of Rienk Offrenga, and the level of knowledge and skill in the art, provide enabling support for claims 1 and 40-81. Appellants will first address the Examiner's assertion that when a previously undescribed mechanism of action is unexpected it is therefore unpredictable for the purposes of enablement. *See*, Office Action mailed September 19, 2002, at pages 2 and 3; Office Action mailed May 21, 2003, at Page 3. Appellants respectfully submit that an unexpected result in not necessarily unpredictable for the purposes of enablement. If the Examiner's assertion were valid, any argument for nonobviousness citing unexpected results (as is permissible under MPEP § 716.02) would automatically lead to a rejection for lack of enablement under 35 U.S.C. § 112, first paragraph. Appellants respectfully submit that the established value of unexpected results in demonstrating nonobviousness would be seriously undermined if any demonstration of unexpected results automatically indicated a finding of unpredictability.

Further, an unexpected result does not necessarily equate to an unpredictable result. If the odds of a particular result occurring are very low, then the occurrence of that result may be unexpected, but it is not unpredictable. In addition, the appellants, persons of knowledge and skill in the art, would not have engaged in the experimentation necessary to demonstrate the unexpected result if they did not first predict the result would occur. Last, once the unexpected results of the invention are described, the results are no longer unpredictable as one could follow

the teachings of the specification and arrive at similar results. As such, appellants respectfully submit, contrary to the Examiner's assertion, that the unexpected results that the give rise to the present invention do not make the present invention unpredictable in terms of enablement.

Appellants further respectfully submit, as to the Examiners first key factor of the rejection, that the specification adequately enables how the antigens that induce unwanted T cell responses are established. Appellants respectfully submit that which antigen a T-cell response is to be reduced (an "unwanted T-cell response") is subjective in nature. The selection of the antigen to which a T-cell response is to be reduced is a decision left to the practitioner of the invention (presumably one of knowledge and skill in the art) and any antigen can be selected (using the knowledge and skill in the art) for use in practicing the present invention. Further, the appellants provide two examples of antigens which were tested to see if a T-cell response to them was reduced. Specifically, Example 4 of the specification (¶ 35) shows the reduction of a T-cell response to the antigens hsp65 protein and p3-13. For the purposes of testing the invention, the T-cell response to hsp65 and p3-13 were "unwanted" as the practitioners of the invention wished to reduce the T-cell response to these antigens. As such, the selection of the "unwanted T-cell response" and the corresponding antigen(s) which induces that T-cell response is placed in the hands of one of skill in and knowledge in the art using selection criteria based on that skill and knowledge. As such, the selection of an "unwanted T-cell response" according to the present invention can be arrived at by one of skill in the art and thus allows one of skill and knowledge to make and use the claimed invention.

In addition, an advantage of the invention is that the exact identity of all possible antigens is not required. Therefore, the invention is not limited to the use of any specific antigen. A person of ordinary skill in the art would recognize that professional antigen presenting cells (APCs)[1], such as dendritic cells, can be loaded with any antigen for which tolerization is desired (*i.e.*, the unwanted immune response). Antigens will be taken up, processed and displayed by MHC class I and II molecules on the surface of the APC to surrounding T-cells (Kampgen *et al.* (1991), Class II Major Histocompatibility Complex Molecules of Murine Dendritic Cells:

[1] Antigen presenting cells (APC) are cells derived from bone marrow and comprise a heterogeneous set of cells, including dendritic cells in lymphoid organs, Langerhans cells in skin, and certain types of macrophages, which present antigens on MHC glycoproteins. BRUCE ALBERTS *ET AL.*, MOLECULAR BIOLOGY OF THE CELL (2nd ed. Garland Publishing, Inc., 1989) pp. 1044-1048, 1045.

Synthesis, Sialylation of Invariant Chain, and Antigen Processing Capacity are Down-Regulated Upon Culture. *Proc. Natl. Acad. Sci. U S A.* 88(8):3014-3018 (discussing the abundant antigen presentation by dendritic cells through MHC molecules)).[2] Thus, loading of an antigen may be accomplished by bringing dendritic cells into contact with any antigen source for which tolerization is desired, ranging from its own internal proteins (as in Figure 5 of the Declaration; Hancock *et al.*, 1996; and Kampgen *et al.*, 1991) or external sources, such as, purified peptides, proteins or cell extracts from grafts/transplants (for example, as in ¶¶ 35 and 43 of the specification). In Example 5 of the Declaration, the dendritic cells are syngeneic (*i.e.*, of the exact same origin and genetic make-up as the graft cells), hence expressing, or loaded with, antigens identical to the graft/transplant antigens.

Furthermore, antigens for specific diseases are known in the art. For example, multiple sclerosis is a demyelinization disease, associated with an autoimmune response to the myelin basic protein. Nicholson *et al.* disclose alterations of an antigen and states that "[m]uch of the experimental work in models of autoimmunity has focused on the immune response to specific peptide ligands (cognate ligands)."[3] In addition, Greten *et al.* disclose that "many HLA-A2-restricted antigens have been identified for human ... autoimmune diseases, and cancer."[4] Finally, Stemme *et al.* disclose autoantigens derived from oxLDL as being of potentially "significant pathogenetic importance in atherosclerosis."[5] Thus, the selection of an antigen for a specific disease is known in the art.

As explained herein, a limiting list of specific antigens is not expressly provided in the disclosure, as the invention is not limited to any specific antigen. In practice, when tolerizing a subject for grafts or transplants it is not even feasible to point out a single specific antigen, as there are typically many reactive antigens. As exemplified in the Declaration, for example, Figure 5, an entire spectrum of antigens derived from a graft/transplant can be displayed on

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[2] Kampgen *et al.*, like Cronstein *et al.*, does not specifically list all possible antigens present in a cell.

[3] Nicholson *et al.* (1998) Heteroclitic Proliferative Responses and Changes in Cytokine Profile Induced by Altered Peptides: Implications for Autoimmunity *Proc. Natl. Acad. Sci. U.S.A.* 95:264-269, 264.

[4] Greten *et al.*, (1998) Direct Visualization of Antigen-specific T Cells: HTLV-1 Tax11-19-specific $CD8^+$ T Cells are Activated in Peripheral Blood and Accumulate in Cerebrospinal Fluid From HAM/TSP Patients, *Proc. Natl. Acad. Sci. U.S.A.* 95:7568-7573, 7572.

[5] Stemme *et al.* (1995) T Lymphocytes from human Atherosclerosis Plaques Recognize Oxidized Low Density Lipoprotein, *Proc. Natl. Acad. Sci. U.S.A.* 92:3893-3897, 3897.

alternatively activated dendritic cells according to the current invention. There simply is no need, nor is it desirable, to select a single antigen in order to reduce an unwanted immune response. Further, the antigens will depend on the genetic make-up of the host (HLA type). Thus, the specification provides the only possible enabling description of the antigens (for example, ¶ 7 of the specification, indicating "that such DC [dendritic cells] loaded with appropriate antigens [as can be selected by one of skill and knowledge in the art] can be exploited as a novel approach for specifically down regulating unwanted T-cell responses *in vivo.*") (emphasis added). Further, specific antigens, be they multiple antigens from an allogeneic graft or transplant or specific antigens such as the myelin basic protein, are known in the art.

In addition, the Declaration under 37 C.F.R. § 1.132 states that "[t]he enclosed summary of the tests as set forth below also demonstrate the practical use of alternatively activated DC for modulation of the alloimmune response and show that these can induce a prolonged skin graft survival even in a complete MHC incompatible donor-recipient combination; and that Example 1 herein was based on Example 4 of the patent application" (page 2 of the Declaration). Further, a person of ordinary skill in the art knows that "[e]ach ... [animal] has a group of genes (the major histocompatibility complex or MHC) which codes for many proteins important for immune function. Among these are a group of proteins located on the plasma membranes of all nucleated cells in an individual's body called human leukocyte associated antigens (HLA antigens) .... Since no two persons (other than identical twins [and inbred laboratory strains, *e.g.*, inbred mice]) have the same MHC genes, no two persons have the same HLA antigens" (ARTHUR J. VANDER, M.D. ET. AL., HUMAN PHYSIOLOGY: THE MECHANISMS OF BODY FUNCTION 621 (Mary Jane Martin and Susan Hazlett eds., McGraw-Hill Book Co. 4th ed. 1985) (emphasis in original) (*see also*, ABUL K. ABBAS, M.B.B.S., ET. AL., CELLULAR AND MOLECULAR IMMUNOLOGY 319 (W. B. Sauders Co., 1991) (both cited in an IDS submitted April 27, 2005, and not considered as the citation lacked the city of publication). Moreover, a person of ordinary skill in the art knows that "[t]he cell-mediated immune system is also mainly responsible for the recognition and destruction, *i.e.*, rejection, of tissue transplants .... [and that] on the surfaces of all nucleated cells of an individual's body are genetically determined antigenic protein molecules known as HLA or histocompatibility antigens. When tissue is transplanted from one individual to another, those

surface antigens which differ from the recipient's are recognized as foreign and are destroyed by sensitized cytotoxic T cells" (*Id.* A. J. VANDER at 626, A. K. ABBAS at 319-320) (emphasis in original). Hence, a person of ordinary skill in the art recognizes that the mice used as the donor and recipient have different HLA antigens, *see*, for example, the Declaration at page 5, which indicates the different HLA types for the mice, and that the surface antigens of the graft, which differ from the recipient's, will be recognized as foreign and be destroyed by the host T cells (*see also*, Hancock *et al.*, 1996, indicating the HLA types for the mice used and presuming that the reader is familiar with the basic concepts of allogenic rejection). Therefore, the "presumptions" of the authors are more "more than an attorney's assertion" (Office Action mailed December 27, 2004, at Page 3) and the inventor's § 1.132 Declaration does address the invention of the instant claims. More specifically, the inventor's Declaration addresses a method for preparing a pharmaceutical composition for reducing an unwanted T-cell response in a host [allogenic graft rejection], said method comprising ... activating said dendritic cells with a means for reducing IL-12p40 production by said dendritic cells (or activating said dendritic cells with a glucocorticoid capable of activating a glucocorticoid receptor) [activating the dendritic cells with dexamethasone]; loading said dendritic cells with (or bringing said dendritic cells into contact with) an antigen against which said T-cell response is to be reduced; and forming a pharmaceutical composition comprising said loaded, activated dendritic cells for administration to the host, as recited in the pending claims (*e.g.*, claims 1 and 40).

The appellants have also provided evidence that the DCs in the example provide the same antigens as the skin graft cells and that an antigen-specific unwanted T cell response [allogenic graft rejection] is reduced (Office Action mailed December 27, 2004, at Page 3). For example, the Declaration states that "prolonged skin graft survival after treatment with alternatively activated H-2^b DC [indicating the HLA type] was **specific** for the H-2^b alloantigens as mice injected with DEX-LPS DC rejected skin grafts from DBA/1 mice (H-2^q) [HLA type different from the antigens loaded on the dendritic cells] in the same time ... as control mice" (page 10 of the Declaration) (emphasis added). Therefore, the Declaration does provide evidence that the DCs provide the same antigens as the skin graft cells, and that the unwanted T cell response, *e.g.*, graft rejection, is reduced.

The Examiner also asserts that the examples of the Declaration are not antigen specific – alloimmune responses are not considered to be antigen-specific responses. Office Action mailed December 27, 2004, at Page 3 The appellants respectfully disagree. As discussed herein, the dendritic cells (DC) were **specific** for the H-2^b alloantigens, thus, the response is specific to the antigens of the graft cells.

The Examiner further asserts that appellants' argument "that professional antigen presenting cells (APCs)[6], such as dendritic cells, can be loaded with any antigen for which tolerization is desired .... Thus, loading of an antigen may be accomplished by bringing dendritic cells into contact with any antigen source for which tolerization is desired, ranging from its own internal proteins (as in Figure 5 of the Declaration; Hancock *et al.*, 1996; and Kampgen *et al.*, 1991) or external sources, such as, purified peptides, proteins or cell extracts from grafts/transplants (for example, as in ¶¶ 35 and 43 of the specification)" (*see*, page 14 of the reply mailed November 21, 2003) is confusing, since it is unclear how DC can be loaded with any antigen ... if the identity of the said antigens has not been established" (Office Action mailed December 27, 2004, at pages 3-4). Appellants respectfully submit that a person of ordinary skill in the art clearly understands how dendritic cells can be loaded with antigens without having to determine the identity of each antigen (*see*, A. K. ABBAS at 319-320).

Moreover, the Examiner has provided no evidence to contradict the Offrenga Declaration. The Federal Circuit, in *In re Zurko*, states that the Board must make its findings based upon the written record. 59 USPQ2d 1693, 1997 (Fed. Cir. 2001). Here, the written record consists of the Offrenga Declaration and its cited art. The Examiner has produced nothing other than his own understand and experience to refute appellants evidence. Accordingly, appellants respectfully request that the Offrenga Declaration be accepted as proof that the transplant models described in the Declaration establish how an unwanted T-cell response can be selected.

Hence, a person of ordinary skill in the art knows "how dendritic cells can be loaded with any antigen for which tolerization is desired if the identity of said antigens has not been established" (Office Action mailed December 27, 2004, at Page 4).

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[6] See footnote 1, *supra*

In addition, the Examiner further asserts that the appellants' statement that antigens for specific diseases are known in the art is a "severe oversimplification" (Office Action mailed December 27, 2004, at Page 4) and that Greten *et al.*, Stemme *et al.* and Nicholson *et al.* do not provide any definitive statements regarding T cells specific for the described antigens being absolutely known to be pathogenic (*Id.*). The appellants respectfully disagree. For example, Greten *et al.* at 7568 state "[i]t has been previously demonstrated that circulating $CD8^+$ cytotoxic T lymphocytes (CTLs) in patients with HAM/TSP react against HTLV-1 protein products, and an immunodominant HLA-A2-restricted epitope (HTLV-1 Tax11–19)," and Stemme *et al.* at 3896 state that their work "strongly suggests that the T-cell response was mounted against an HLA-DR-restricted, processed antigen derived from ox-LDL." The Office then goes on to discuss clinical difficulties encountered in the treatment of multiple sclerosis by reducing the number of T cells specific for the known antigen, myelin basic protein (MBP). Hence, the Office acknowledges that MBP is a known antigen for multiple sclerosis. Regardless of whether or not the authors "tortured" their analysis of the data (page 4 of the Office Action), the antigen is known in the art (*see*, Zhang *et al.* at 212).

In view of the foregoing, appellants respectfully submit that the selection of an "unwanted T-cell response" according to the present invention can be arrived at by one of skill in the art and thus allows one of skill and knowledge to make and use the claimed invention.

In regards to the Examiners second key factor, the Examiner states that the specification "does not reasonably provide enablement for, *in vivo* or *in vitro* induction of non- responsiveness of polyclonal T cells to any undefined antigen or the *in vivo* induction of non-responsiveness when an 'unwanted T-cell response' is ongoing" (emphasis added; Office Action mailed May 21, 2003, at Page 3). The appellants respectfully submit that the Examiner appears to indirectly put forward the position that the *in vitro* data of the specification does not support claims to *in vivo* uses.[7] The appellants have described how the invention functions using *in vitro* working examples. However, the Examiner acknowledges enablement of only *in vitro* use of the invention. The appellants respectfully submit that the specification is enabling for both *in vitro*

[7] The appellants submit that a requirement for *in vivo* data to support *in vivo* use is contrary to established law (*see* MPEP § 2164.02, citing *In re Brana*, 51 F.3d 1560, 1566, 34 USPQ2d 1436, 1441 (Fed Cir. 1995); Fujikawa v. Wattanasin, 93 F.3d 1559, 1565-1566, 39 USPQ.2d (BNA) 1895 (Fed. Cir. 1996)).

and *in vivo* induction of non-responsiveness of polyclonal T cells.[8] As the Office bears the burden of presenting reasons for the lack of an enabling correlation between *in vitro* and *in vivo* results, in the absence of any such reasoning, the appellants assume that no challenge to the correlation of the *in vitro* data to use *in vivo* is intended. Thus, the appellants submit that the *in vitro* data provided in the specification supports and enables *in vivo* use of the claimed compositions. Furthermore, the appellants respectfully submit that the *in vitro/in vivo* correlation is firmly established by the executed Declaration, submitted herewith.

In addition, the appellants submit that the Examiner's second key factor is answered in the Declaration. The examples provided in the Declaration demonstrate unequivocally the use of the alternatively stimulated dendritic cells as a pharmaceutical composition *in vivo* (Office Action mailed May 21, 2003, at Page 3). The alternatively activated dendritic cells are capable of inducing a prolonged skin graft survival when administered as a pharmaceutical composition *in vivo* to mice having undergone a skin graft with an incompatible donor-recipient combination. Figures 5a and 5b of the Declaration show a striking difference in skin graft survival. In Figure 5a the administered dendritic cells are of the same type C57BL/6 (H-2^b) as the graft (*i.e.*, carrying and displaying the same antigens), demonstrating that alternatively activated dendritic cells displaying antigens identical to the antigens displayed on the graft cells are capable of tolerizing a subject and result in prolonged (about doubled) graft survival. The Examiner alleges that no antigen loaded DCs were used in the disclosed experiments. The appellants would again like to stress that the activated antigen presenting dendritic cells in this experiment do display antigens. In particular, the dendritic cells present the same antigens as the skin graft cells, which

[8] The Office Actions, while rejecting the claims for an alleged lack of written description seem to the appellants to be directed to enablement of glucocorticoids other than dexamethasone. Cronstein *et al.*, (1992) A Mechanism for the Anitiinflammatory Effects of Corticosteroids: The Glucocorticoid Receptor Regulates Leukocyte Adhesion to Endothelial Cells and Expression of Endothelial-leukocyte Adhesion Molecule 1 and Intercellular Adhesion Molecule 1, *Proc. Nat. Acad. Sci. U.S.A.* 89:9991-9995) (of record in the case) shows the use of dexamethasone and references comparison to the weaker glucocorticoid, cortisol, as an example of glucocorticoids. The authors of this paper conclude that "antagonism by dexamethasone ... is a specific instance of the general biological principle that the glucocorticoid receptor is a hormone-dependent regulator of transcription" (Cronstein *et al.*, summary). Cronstein *et al.* recognize that the results that they obtained with the example dexamethasone are applicable to the entire glucocorticoid class. Likewise, the appellants used dexamethasone as a representative of all glucocorticoids and apply those results to glucocorticoids in general. In the absence of reasoning why results obtained with dexamethasone would not be applicable to glucocorticoids in general, the appellants submit that the data enables all glucocorticoids and that the express word "glucocorticoids" provides adequate written description of "glucocorticoids."

are also of C57BL/6 (H-2^b) origin, thereby inducing tolerization to these antigens (*see also*, Hancock *et al.*, 1996). The recipient/host mice are of a different type, BALB/c (H-2^d), than the donor and therefore will normally immunologically react to the allogeneic C57BL/6 graft. *Id.* Thus, dendritic cells loaded with C57BL/6, type H-2^b, antigens, function as a pharmaceutical composition *in vivo*, as described in the specification, and the specification teaches a person of ordinary skill in the art how the antigens that induce unwanted T cell responses are established (*e.g.*, graft derived).

In Figure 5b, a different skin graft was used (DBA/1 (H-2^q) origin). Indeed, here the C57BL/6-antigen displaying C57BL/6 dendritic cells (the graft and dendritic cells are not syngeneic) were ineffective in prolonging the survival of the DBA/1 graft (*i.e.*, no tolerization of the graft was observed). Thus, Figure 5a illustrates the effectiveness of the claimed method and *in vivo* function of the invention, as described in the specification, confirming the *in vivo* applicability of the appellants' *in vitro* tests (for example, Example 4). Figure 5b illustrates the specificity of the claimed method.

The Office asserts that no antigen-loaded DCs were used in the disclosed experiments [of the Declaration]. Hancock *et al.* (1996), Costimulatory function and expression of CD40 ligand, CD80, and CD86 in vascularized murine cardiac allograft rejection., *Proc. Natl. Acad. Sci. U S A.* 93(24):13967-13972, describes graft versus host rejection with C57BL/6 (H-2b) and BALB/c (H-2d) mice through CD40L binding. As will be noted by review of Hancock *et al.*, the authors of the reference do not list the source or identity of all of the antigens, other than by reference to the particular mice (for example, C57BL/6 (H-2^b) and BALB/c (H-2^d), page 13967, second column), but simply refer to allograft rejection (for example, Figures 1-3 and Table 2). Thus, Hancock *et al.* presumes that a person of ordinary skill in the art would recognize the source of the antigens (the allogeneic mice). Likewise, the Declaration does not expressly state the source of the antigens. The source of the antigens is presented in the form of a description of the allogeneic mice C57BL/6 (H-2^b) and BALB/c (H-2^d).

Moreover, the Examiner has provided no evidence to contradict the Offrenga Declaration. The Federal Circuit, in *In re Zurko*, states that the Board must make its findings based upon the written record. 59 USPQ2d 1693, 1997 (Fed. Cir. 2001). Here, the written record consists of the Offrenga Declaration and its cited art. The Examiner has produced nothing

other than his own understand and experience to refute appellants evidence. Accordingly, appellants respectfully request that the Offrenga Declaration be accepted as proof that the transplant models described in the Declaration establishes how the *in vitro* experimentation outlined in the specification is directly applicable to *in vivo* results.

Last, the Examiner asserts, in the Office Action mailed July 26, 2005, at Page 5, that no nexus has been established between a reduction in IL12p40 production and a reduction in an unwanted T cell response. Further, the Examiner asserts "as no cause an effect has been tested for, and thus, not established, it is just as, or more, likely that the reduction of IL12p40 is just an artifact, or at most a marker for the reduction of an antigen specific T cell response." *Id.*

Appellants respectfully submit that the nexus between IL12p40 production and a reduction in unwanted T cell response exists in the specification. Specifically, Example 3 (¶ 34) indicates that a Key feature of CD40 triggered DC for initiating T-cell immunity resides in their ability to produce the proinflammatory cytokine IL-12. The specification therein cites the work of Cella *et al.*, J Exp Med 184: 747, 1996, Koch *et al.*, J Exp Med 184: 741, 1996, and de Saint Vis *et al.*, J Immunol 160: 1666, 1998 as support for this statement. Further, when the findings of Example 3 are combined with those of Example 4, it would be apparent to one of skill in the art that the corresponding reduction of IL12p40 production in Example 3 leads to the reduction of a T-cell response presented in Example 4. As such, appellants respectfully submit that one of skill in the art would agree that a nexus exists between IL12p40 production and a reduction in unwanted T cell response.

Further, an inventor need not comprehend the scientific principles upon which the practical effectiveness of the invention rests. *Fromson v. Advance Offset Plate, Inc.*, 720 F.2d 1565, 219 USPQ 1137 (Fed. Cir. 1983). As such, even if, as asserted by the Examiner, the reduction of IL12p40 is just a marker for the reduction of an antigen specific T cell response, appellants need not understand exactly how the invention works. It is sufficient that the invention does work and that the specification, in combination with the knowledge and skill in the art, allows one to make and use the invention.

In view of the foregoing, appellants respectfully submit that the specification does disclose, in the only feasible way, precisely how the antigens that induce unwanted T cell responses are established. In addition, the specification describes a mechanism of the invention

using *in vitro* data which correlates and enables use *in vivo*. Thus, a person of ordinary skill in the art, who would recognize the source of the antigens from the description in the specification, is enabled to practice the invention *in vitro* and *in vivo*. Therefore, as one of skill in the art, using the knowledge and skill in the art, can practice the present invention, including the selection of an "unwanted T-cell response," appellants respectfully submit that claims 1, and 40-81 are enabled under 35 U.S.C. § 112, first paragraph. *See*, *In re Fisher*, 4127 F.2d 833, 839, 166 USPQ 18, 24 (CCPA 1970). Consequently, appellants respectfully request that the rejections of claims 1 and 40-81 under 35 U.S.C., first paragraph, for lack of enablement be withdrawn and the claims allowed.

(8) CLAIMS APPENDIX

1. A method for preparing a pharmaceutical composition for reducing an unwanted T-cell response in a host, said method comprising:

culturing peripheral blood monocytes from said host to differentiate into dendritic cells;

activating said dendritic cells with a means for reducing IL-12p40 production by said dendritic cells;

loading said dendritic cells with an antigen against which said T-cell response is to be reduced; and

forming a pharmaceutical composition comprising said loaded, activated dendritic cells for administration to said host.

40. A method for preparing a pharmaceutical composition for reducing an unwanted T-cell response in a host against an antigen, said method comprising:

culturing peripheral blood monocytes from said host to differentiate into dendritic cells;

activating said dendritic cells with a glucocorticoid capable of activating a glucocorticoid receptor;

bringing said dendritic cells into contact with an antigen against which said T-cell response is to be reduced; and

forming a pharmaceutical composition comprising said loaded, activated dendritic cells.

41. The method according to claim 40, further comprising activating a CD40 receptor on said dendritic cells.

42. The method according to claim 41, wherein activating the CD40 receptor comprises incubating the dendritic cells with a substance selected from the group consisting of a CD8-40L fusion protein, a trimeric form of CD40L consisting of CD40L molecules to which a modified leucine zipper has been attached, anti-CD40 antibodies, and cells that express CD40L.

43. The method according to claim 40, wherein bringing said dendritic cells into contact with an antigen comprises incubating said dendritic cells with at least one peptide representing at least one antigen of interest before activating said dendritic cells with said substance capable of activating the glucocorticoid receptor.

44. The method according to claim 40, wherein bringing said dendritic cells into contact with an antigen comprises incubating said dendritic cells with cells containing at least one antigen of interest before activating said dendritic cells with said substance capable of activating the glucocorticoid receptor .

45. The method according to claim 40, wherein bringing said dendritic cells into contact with an antigen against which said T-cell response is to be reduced comprises loading said dendritic cells with at least one synthetic peptide representing at least one antigen of interest after

activating said dendritic cells with said substance capable of activating the glucocorticoid receptor .

46. The method according to claim 40, wherein activating said dendritic cells with said substance capable of activating the glucocorticoid receptor comprises activating said dendritic cells such that said dendritic cells secrete interleukin-10.

47. The method according to claim 40, wherein said T-cell is a T-helper cell.

48. The method according to claim 40, wherein bringing said dendritic cells into contact with an antigen comprises incubating said dendritic cells with a cell homogenate containing at least one antigen of interest before activating said dendritic cells with said substance capable of activating the glucocorticoid receptor.

49. The method of claim 40, further comprising incubating the dendritic cells with a substance selected from the group consisting of lipopolysaccharide (LPS) and polyI/C.

50. The method of claim 40, wherein said glucocorticoid capable of activating the glucocorticoid receptor comprises dexamethasone.

51. A method for obtaining a dendritic cell capable of tolerizing a T-cell for an antigen, comprising:

providing said dendritic cell with a substance capable of activating a glucocorticoid receptor;

activating said dendritic cell; and

providing said dendritic cell with said antigen, wherein said dendritic cell is capable of tolerizing a T-cell for said antigen.

52. The method according to claim 51, wherein providing said dendritic cell with the substance capable of activating a glucocorticoid receptor is in vitro.

53. The method according to claim 51, wherein providing said dendritic cell with said substance capable of activating the glucocorticoid receptor comprises providing a precursor of said dendritic cell with said substance capable of activating the glucocorticoid receptor in vitro.

54. The method according to claim 51, wherein said-substance capable of activating the glucocorticoid receptor comprises dexamethasone.

55. The method according to claim 52, wherein said substance capable of activating the glucocorticoid receptor enhances secretion of IL-10 by said dendritic cells.

56. A method for preparing an isolated dendritic cell, said method comprising:

isolating peripheral blood monocytes from a subject;

culturing the peripheral blood monocytes to differentiate into dendritic cells;

activating the dendritic cells with a glucocorticoid;
loading the dendritic cells with an antigen; and
isolating said loaded, activated dendritic cells.

57. The method according to claim 56, wherein the glucocorticoid is dexamethasone.

58. The method according to claim 56, wherein loading said dendritic cells with an antigen comprises loading said dendritic cells with an antigen defined by a response of a T-cell.

59. The method according to claim 56, wherein the antigen comprises an allogeneic antigen.

60. The method according to claim 59, wherein the glucocorticoid is dexamethasone.

61. The method according to claim 60, wherein loading said dendritic cells with an antigen comprises contacting said dendritic cells with cells derived from a graft or transplant donor.

62. The method according to claim 61, wherein the dendritic cells are derived from the graft or transplant recipient.

63. The method according to claim 56, further comprising incubating the dendritic cells with a substance selected from a group consisting of a CD8-40L fusion protein, a trimeric form of CD40L consisting of CD40L molecules to which a modified leucine zipper has been attached, anti-CD40 antibodies, and cells that express CD40L.

64. A method for preparing a dendritic cell capable of tolerizing a T-cell, said method comprising:
culturing peripheral blood monocytes to differentiate into dendritic cells;
activating the dendritic cells with dexamethasone; and
loading the dendritic cells with an antigen which is MHC-matched to a clonal T-cell, wherein the dendritic cells are capable of tolerizing the clonal T-cell in vitro to the antigen.

65. A method for preparing a dendritic cell for tolerizing a T-cell in a graft or transplant recipient, said method comprising:
culturing peripheral blood monocytes from said graft or transplant recipient to differentiate into dendritic cells;
activating said dendritic cells; and
loading said dendritic cells with an antigen against which said T-cell is to be tolerized.

66. The method according to claim 65, wherein activating said dendritic cells comprises administering a glucocorticoid.

67. The method according to claim 66, wherein activating said dendritic cells comprises administering dexamethasone.

68. The method according to claim 65, wherein loading said dendritic cells with an antigen comprises contacting said dendritic cells with cells derived from a graft or transplant donor.

69. A method for preparing a pharmaceutical composition for reducing an unwanted T-cell response to an antigen in a host, said method comprising:

culturing peripheral blood monocytes from said host to differentiate into dendritic cells *in vitro*;

contacting said dendritic cells *in vitro* with an antigen against which said T-cell response is to be reduced, thereby loading said dendritic cells with the antigen;

contacting said dendritic cells with dexamethasone;

activating the CD40 receptor on said dendritic cells; and

forming a pharmaceutical composition comprising said loaded, activated dendritic cells.

70. The method according to claim 69, wherein activating the CD40 receptor comprises culturing the dendritic cells with a substance selected from the group consisting of a CD8-40L fusion protein, a trimeric form of CD40L comprising CD40L molecules having a modified leucine zipper covalently attached to said CD40L molecules, anti-CD40 antibody, and cells that express CD40L.

71. The method according to claim 69 further comprising contacting the dendritic cells with lipopolysaccharide (LPS) or polyI/C.

72. The method according to claim 69, comprising contacting said dendritic cells *in vitro* with an antigen against which said T-cell response is to be reduced before contacting said dendritic cells with dexamethasone.

73. The method according to claim 72, wherein contacting said dendritic cells *in vitro* with an antigen against which said T-cell response is to be reduced comprises co-culturing said dendritic cells and cells containing at least one antigen of interest.

74. The method according to claim 69, comprising contacting said dendritic cells *in vitro* with an antigen against which said T-cell response is to be reduced after contacting said dendritic cells with dexamethasone.

75. The method according to claim 74, wherein contacting said dendritic cells *in vitro* with an antigen against which said T-cell response is to be reduced comprises contacting said dendritic cells with at least one isolated peptide having at least one antigenic region of interest.

76. The method according to claim 72, wherein contacting said dendritic cells *in vitro* with an antigen against which said T-cell response is to be reduced comprises contacting said dendritic cells with a cell homogenate containing at least one antigen of interest.

77. A method for obtaining a dendritic cell capable of tolerizing a T-cell for an antigen, the method comprising:

contacting a dendritic cell with dexamethasone *in vitro*;

activating the dendritic cell through the CD40 receptor; and

contacting the dendritic cell with an antigen, thereby loading the dendritic cell with the antigen, and forming a dendritic cell capable of tolerizing a T-cell for the antigen.

78. The method according to claim 77, wherein the dendritic cell is derived from a graft or transplant donor.

79. The method according to claim 77, further comprising:

isolating peripheral blood monocytes from a subject;

culturing the peripheral blood monocytes to differentiate into dendritic cells;

incubating the dendritic cells with a substance selected from the group consisting of a CD8-40L fusion protein, a trimeric form of CD40L comprising CD40L molecules having a modified leucine zipper covalently attached to said CD40L molecules, anti-CD40 antibodies, cells that express CD40L, lipopolysaccharide (LPS) and polyI/C; and

isolating the dendritic cell.

80. The method according to claim 79, wherein contacting the dendritic cell with the antigen comprises contacting the dendritic cell with cells derived from a graft or transplant donor.

81. The method according to claim 79, wherein the peripheral blood monocytes are derived from the graft or transplant recipient.

(9) EVIDENCE APPENDIX

(A) Declaration Under 37 C.F.R. § 1.132 of Rienk Offringa. Noted as entered into the record by the Examiner in the Final Office Action mailed December 27, 2004, at Page 2.

(B) Goodman *et al.*, Goodman and Gilman's The Pharmacological Basis of Therapeutics, (Seventh Ed.) Ch 63 Adrenocorticotrophic Hormone: Adrenocortical Steroids and their Synthetic Analogs: Inhibitors of Andrenocortical Steroid Biosynthesis, page 1464. First presented in the Office Action Response mailed October 29, 2001, and responded to by the Examiner in Office Action mailed September 19, 2002.

(C) Cronstien B.M., Kimmel S.C, Levin R.I., Martinuik F., and Weismann G: A mechanism for the anti-inflammatory effect of corticosteroids: the glucocorticoid receptor regulates leukocyte adhesion to endothelial cells and expression of endothelial-leukocyte adhesion molecule 1 and intercellular adhesion molecule 1. (1992) Proc. Natl. Acad. Sci. USA 89:9991-9995. Submitted in an IDS mailed November 21, 2003; noted as considered by the Examiner in the Form 1449 initialed December 22, 2004.

(D) Kampgen *et al.* (1991), Class II Major Histocompatibility Complex Molecules of Murine Dendritic Cells: Synthesis, Sialylation of Invariant Chain, and Antigen Processing Capacity are Down-Regulated Upon Culture. *Proc. Natl. Acad. Sci. U S A.* 88(8):3014-3018. Submitted in an IDS mailed November 21, 2003; noted as considered by the Examiner in the Form 1449 initialed December 22, 2004.

(E) Bruce Alberts et al., Molecular Biology of The Cell (2nd ed. Garland Publishing, Inc., 1989) pp. 1044-1048. First submitted in an IDS mailed November 21, 2003. Resubmitted in an IDS mailed April 27, 2005.

(F) Nicholson *et al.* (1998) Heteroclitic Proliferative Responses and Changes in Cytokine Profile Induced by Altered Peptides: Implications for Autoimmunity *Proc. Natl. Acad. Sci. U.S.A.* 95:264-269, 264. Submitted in an IDS mailed

November 21, 2003; noted as considered by the Examiner in the Form 1449 initialed December 22, 2004.

(G) Greten *et al.*, (1998) Direct Visualization of Antigen-specific T Cells: HTLV-1 Tax11-19-specific $CD8^+$ T Cells are Activated in Peripheral Blood and Accumulate in Cerebrospinal Fluid From HAM/TSP Patients, *Proc. Natl. Acad. Sci. U.S.A.* 95:7568-7573, 7572. Submitted in an IDS mailed November 21, 2003; noted as considered by the Examiner in the Form 1449 initialed December 22, 2004.

(H) Stemme *et al.* (1995) T Lymphocytes from human Atherosclerosis Plaques Recognize Oxidized Low Density Lipoprotein, *Proc. Natl. Acad. Sci. U.S.A.* 92:3893-3897, 3897. Submitted in an IDS mailed November 21, 2003; noted as considered by the Examiner in the Form 1449 initialed December 22, 2004.

(I) Arthur J. Vander, M.D. Et. Al., Human Physiology: The Mechanisms of Body Function 621 (Mary Jane Martin and Susan Hazlett eds., McGraw-Hill Book Co. 4^{th} ed. 1985). Submitted in an IDS mailed April 27, 2005.

(J) Abul K. Abbas, M.B.B.S., Et. Al., Cellular and Molecular Immunology 319 (W. B. Sauders Co., 1991). Submitted in an IDS mailed April 27, 2005.

(K) Zhang *et al.* (2002) T cell vaccination in Multiple Sclerosis: results of a preliminary study, *J. Neurol.* 249:212-218. Cited by the Examiner in Office Action mailed December 27, 2004.

(L) Hancock *et al.* (996) Costimulatory function and expression of CD40 ligand, CD80, and CD86 in vascularized murine cardiac allograft rejection, *Proc. Natl. Acad. Sci. U.S.A.* 93:13967-13792. Submitted in an IDS mailed November 21, 2003; noted as considered by the Examiner in the Form 1449 initialed December 22, 2004.

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Rea et al.

Serial No.: 09/666,430

Filed: September 21, 2000

For: DENDRITIC CELL ACTIVATED IN THE PRESENCE OF GLUCOCORTICOID HORMONES ARE CAPABLE OF SUPPRESSING ANTIGEN-SPECIFIC T CELL RESPONSES

Confirmation No.: 6289

Examiner: G. Ewoldt

Group Art Unit: 1644

Attorney Docket No.: 2183-4205.1US

Declaration Under 37 C.F.R. § 1.132

I, ROTTRINGA a citizen of the Netherlands, hereby declare and state as follows:

That I received a degree in BIOLOGY from LEIDEN UNIVERSITY, in 1985; and a Doctor of Philosophy in BIOMEDICAL SCIENCES from LEIDEN UNIVERSITY, in 1991;

That I am among the joint inventors of the referenced patent application;

That I conducted (or worked directly with) the series of tests related to this Declaration;

That the enclosed summary of the tests as set forth below demonstrate DEX-treated DC can exert a potent immunoregulatory effect on Th1-immunity at two levels: directly through the suppression of proliferation and IFN γ secretion by both naive and memory-type T cells, and

indirectly through the mobilization of IL-10-secreting T cells the presence of which can also suppress proliferation and IFN γ secretion by Th1 cells. The enclosed summary of the tests as set forth below also demonstrate the practical use of alternatively activated DC for modulation of the alloimmune response and show that these can induce a prolonged skin graft survival even in a complete MHC incompatible donor-recipient combination; and

That Example 1 herein was based on Example 4 of the patent application.

I further hereby declare that the enclosed summary of the tests as set forth below correctly reflect the hereinafter described materials, methods, procedures, and results of those tests.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A: Pretreatment with DEX inhibits the phenotypic change induced by LPS triggering of DC. Immature DC were cultured for 24 hours in the absence or the presence of 10^{-6} M DEX and activated with LPS (10 μg/ml) for 48 hours. Flow cytometric analysis showing the immature D1 cell line (immature DC), the LPS triggered mature DC (LPS) and the DEX pretreated LPS matured DC (DEX-LPS).

FIG. 1B: DEX exposed or control immature DC were left in culture without further treatment or stimulated with LPS. Culture supernatants were harvested 48 hours later and IL-12 secretion was analyzed by ELISA. Data are derived from 3 representative independent experiments.

FIG. 2: Pretreatment with DEX impairs the stimulatory capacities of DCs matured with LPS. Allogeneic mixed lymphocyte culture of BALB/c splenocytes with different numbers of DEX treated immature DCs (DEX), mature DCs (LPS), DEX treated mature DCs (DEX-LPS) as compared to untreated immature DCS. The IFN-γ production (B) was measured in supernatants taken after 48 hours and the proliferative response (A) was analyzed at 66 hours. Data are derived from 3 representative independent experiments.

FIG. 3: *In vivo* effects of alloreactive splenocytes by intravenous (iv) injection of immature DCs and treated DCs into allogeneic mice.

A) The proliferation of different numbers of responding splenocytes after *in vitro* restimulation with C57BL/6 splenocytes is shown after no treatment or after treatment with

either immature DC, DEX treated immature DCs (DEX), mature DCs (LPS), DEX treated mature DCs (DEX-LPS).

B) shows the IFN-γ production by the responding splenocytes at 48 hours after restimulation.

Data are derived from 2 representative independent experiments.

FIG 4: *In vivo* effects of alloreactive splenocytes by subcutaneous (sc) injection of immature DCs and treated DCs into allogeneic mice.

A) The proliferation of different numbers of responding splenocytes after *in vitro* restimulation with C57BL/6 splenocytes is shown after no treatment or after treatment with either immature DC, DEX treated immature DCs (DEX), mature DCs (LPS), DEX treated mature DCs (DEX-LPS).

B) shows the IFN-γ production by the responding splenocytes at 48 hours after restimulation.

C) shows the number of IL-10 producing cells in case the responding splenocytes were restimulated after 7 days *in vitro* stimulation with C57BL/6 splenocytes. Data are derived from 3 representative independent experiments.

FIG 5A: Graft survival of C57BL/6 (H-2^b) donor skin in untreated BALB/c (H-2^d) mice and in mice receiving 7 days before transplantation a sc injection of 1 x 10^6 mature DC (H-2^b) (LPS) or DEX treated mature DC (DEX-LPS). A significantly prolonged graft survival was found in DEX-LPS treated mice compared to mature DC treated mice ($p=0.039$).

FIG 5B: Graft survival of DBA/1 (H-2^q) donor skin in untreated BALB/c (H-2^d) mice and in mice receiving 7 days before transplantation a sc injection of 1 x 10^6 mature DC (H-2^b) (LPS) or DEX treated mature DC (DEX-LPS). No significant prolonged graft survival was found in DEX-LPS treated mice compared to mature DC treated mice ($p=0.92$).

FIG. 7: Dex-treated DC preferentially induce T cells secreting IL-10 instead of IFN γ. Non-adherent PBMC were cultured with either allogeneic CD40-triggered DC or allogeneic DEX-treated CD40-triggered DC. Supernatants were harvested at day 5 and used for measurement of IFN γ and IL-10 by ELISA.

FIG 8: T cells pretreated with modulated DC (DEX-DC) inhibit, in a dose-dependent fashion, the proliferation and cytokine production of alloreactive T cells. Alloreactive T cell

cultures were separately generated through primary stimulation of non-adherent PBMC (donor X) during 10 days with either mature DC or DEX-DC (donor Y), after which viable T cells were isolated and counted. The resulting T cell cultures are designated as T-allo and T-dex. Subsequently, a secondary stimulation of T-allo cells was performed in the presence of mature DC (Donor X) as well as in the presence of titered amounts of T-dex. As a control, secondary stimulation was performed by mixing in titered amounts of T-allo cells. (A) Proliferation was determined after 48 hours of culture by addition of ^{3}H-thymidine for the final 16 hours. (B) Supernatants, harvested from the cultures before addition of ^{3}H-thyxnidine, were used for measurement of IFN-γ production. A representative result of three independent experiments is shown.

EXAMPLE 1

DEX-treated CD40-triggered DC are capable of suppressing Th1-type immunity

Further examination of the T cell responses induced in an allogeneic MLR by CD40-triggered DC versus DEX-treated DC (FIG. 5 of pending application) learned that induction by Dex-treated DC did not merely alter the magnitude of the alloreactive T cell response, but profoundly affected the cytokine production by the T cells stimulated. Whereas the cytokine profile of T cells induced by CD40-triggered DC primarily featured the Th1-type cytokine IFN γ, that of T cells induced by DEX-treated DC was dominated by the immunoregulatory cytokine IL-10 (FIG. 7 attached hereto). This observation prompted us to test whether the T cells induced by DEX-treated DC could themselves exert immunoregulatory properties towards T cell proliferative capacity and IFN γ secretion. Indeed, DEX-DC educated T cells, when mixed in with secondary allogeneic MLR cultures in the presence of CD4-triggered DC, were capable of strongly inhibiting the proliferation and IFN γ production by already primed alloreactive T cells.

Taken together our data demonstrate that DEX-treated DC can exert a potent immunoregulatory effect on Th1-immunity at two levels: directly through the suppression of proliferation and IFN γ secretion by both naive and memory-type T cells, and indirectly through the mobilization of IL-10-secreting T cells the presence of which can also suppress proliferation and IFN γ secretion by Th1 cells.

EXAMPLE 2

DEX-treated activated DC suppress anti-transplant immunity in vivo

Treatment of immature DC with an activating trigger in the presence of a glucocorticoid hormone results in DC maturation through an alternative maturation pathway. We demonstrate in a mouse transplantation model that such alternatively matured DC can successfully be exploited for the induction of donor-specific transplantation tolerance *in vivo*.

Materials and Methods

Mice

Female BALB/c (H-2^d), C57BL/6 (B6; H-2^b) and CBA/Ca (H-2^k) mice were obtained from IFFA Credo (Paris, France). B6.C-$H2^{bml}$/ByJ (class I K^b mutant phenotype) were purchased from The Jackson Laboratories (Bar Harbor, ME, USA). Mice were maintained under specific pathogen-free conditions and used at 6-10 weeks of age.

Cell lines

D1 cell line, a long-term growth-factor dependent immature splenic DC line derived from B6 mice, was cultured as described (Winzler et al, 1997, Maturation stages of mouse dendritic cells in growth factor-dependent long-term cultures, *J. Exp. Med.* 185: 317). Both floating and adherent D1 cells (detached using 2 mM EDTA) were collected and used.

Treatment of DC

The D1 cells were pretreated with dexamethasone (DEX) 10^{-6} M for 24 hrs, after which LPS or nothing was added to the culture for another 48 hours. D1 treated with LPS only (for 48 hours) were also used. Both DEX and LPS (of *E. coli* (Serotype 026:B6)) were purchased from Sigma-Aldrich. After treatment, supernatants were analyzed for the presence of IL-10 and /or IL-12.

Antibodies and cell surface immuno-fluorescence

The following Abs were purchased from PharMingen: FITC-coupled anti CD86 (B7.2), PE-coupled anti CD80 (B7.1), PB-coupled anti-CD40 and PB coupled anti-I-$A^{b/d}$ (M5/114,

MHC class II). Staining was carried out at 4°C for 30 min. Stained cells were analyzed using a FACScan® flow cytometer equipped with CellQuest software (Becton Dickinson).

Cytokine analysis

Harvested supernatants were tested for IL-12 p40/p70, IL-10 or IFN-γ content using a standard sandwich ELISA. Coating Ab: rat anti-mouse IL-12 p40/p70 mAb (clone C15.6, PharMingen), rat anti-mouse IL-10 mAb (clone JES5-2A5, PharMingen) or rat anti-mouse IFN-γ mAb (clone R4-6A2, PharMingen). Detection Ab: biotinylated rat anti-mouse IL-12 p40/p70 mAb (clone C17.8, PharMingen), biotinylated rat anti-mouse IL-10 mAb (clone SXC-1, PharMingen) or biotinylated rat anti-mouse IFN-γ mAb (clone XMG1.2, PharMingen). Streptavidin-HRP and ABTS (Sigma-Aldrich) were used as enzyme and substrate, respectively. OD405 was read by an ELISAreader (Wallac, Turku, Finland).

Proliferation assays

To study alloreactivity, splenocytes (0.5 or 1 x 10^5 cells/well) of BALB/c mice were cocultured with irradiated (30 Gy, two-fold dilutions from 2 x 10^4 cells/well) D1 cells or splenocytes (30 Gy, 1 x 10^5 cells/well). Immature DC pretreated with DEX (DEX), DEX-pretreated immature DC subsequently activated with LPS (DEX-LPS) and LPS matured DC (LPS) were used as APC in the stimulation assays. The cells were plated out in U-bottom 96-well plates (Costar, Cambridge, MA, USA) in Iscove's (IMDM, BioWhittaker) containing 8% heat-inactivated Fetal Calf Serum (Greiner, Alphen, The Netherlands), 100 IU/ml penicillin, 2mM L-glutamin and 20 μM 2-ME. Supernatant was harvested after 48 hours and stored at 20°C. Wells were pulsed with 1 μCi ^{3}H-thymidine (Amersham International, Amersham, UK) and the cultures harvested onto glass fiber filters 18 hours later. Proliferation was measured as ^{3}H-thymidine incorporation by liquid scintillation spectroscopy using a betaplate (Wallac, Turku, Finland).

In vivo treatment with modulated DC analyzed by in vitro alloreactivity

After washing, 10^6 D1 cells were injected intravenously (iv) or subcutaneously (sc) in BALB/c mice in PBS with 0.5% BSA. After 7 days, spleen cells were used for detection of

alloreactive cellular responses (proliferation and cytokine analysis) by *in vitro* stimulation with splenocytes from syngeneic mice (BALB/c), from donor mice (C57BL/6) or from third party mice (CBA/Ca).

Elispot analysis

For Elispot analysis 1 x 10^6 splenocytes (from *in vivo* treated mice) were incubated with 1 x 10^6 C57BL/6 splenocytes in a 24-well plate (Costar) in Iscove's containing 8% heat-inactivated Fetal Calf Serum, 100 IU/ml penicillin, 2mM L-glutamin and 20 μM 2-ME days. The cells were harvested and incubated (at either 1 or 2 x 10^5 cells/well) with irradiated (3000 rad) splenocytes (1 x 10^5 cells/well) from C57BL/6, medium or con A controls during 24 hours in a plate (MAHA S45 10, Millipore) that was precoated with 5 μg/ml antibody (IFN-γ: R4-6A2, IL-10: JES5-2A5). Next, the wells were washed and the detection antibody was added at 0.3 μg/ml (IFN-γ: XMG1.2-biotin, IL-10: SXC-1-biotin) and incubated for 2 hours at room temperature. After another washing step, the conjugate (extravidin alkalin phospatase, Sigma E2636) was added and incubated for 1 hour at room temperature. After washing, the substrate was added and incubated for 10 minutes at room temperature, after which the reaction was stopped with tap water. Analysis of spots was performed by using a BioReader 3000 Pro (BioSys, Karben, Germany).

In vivo treatment with modulated DC analyzed by skin transplantation

After washing, 10^6 D1 cells were injected iv or sc in BALB/c mice in PBS with 0.5% BSA. After 7 days, mice were transplanted on the tail with skin grafts derived from the tail from donor mice.

The skin grafts were protected with a glass pipe of 4.5 cm long, which was kept on the tail for 7 days. Beside this protection, little irritation (and therefore inflammation) was observed because of the fact that the mice were kept on individual basis in cages with a high tech artificial bedding (Omega-Dri) instead of normal sawdust. Graft survival was followed by daily visual inspection. Scoring was performed by comparing with syngeneic grafts and was based on redness, crust-forming and the presence of hairs. The grafts were scored as rejected when they were fully necrotic or fallen off. Statistical analysis was performed using the log rank test.

RESULTS

Characteristics of alternatively activated dendritic cells (phenotype and cytokine production)

A typical FACS profile of immature DC and the influence of DEX treatment and LPS triggering on these DCs can be seen in FIG. 1A. DC matured with LPS showed significant up-regulation of CD86, CD40 and MHC class II (middle panel), when compared to immature DC, whereas DC pretreated with DEX and subsequently matured with LPS (DEX-LPS) did not show up-regulation of CD86 and only marginal up-regulation of CD40 and even a lowered expression of MHC class II (lower panel). We investigated whether DEX affected the production of the pro-inflammatory cytokine IL-12. As shown in FIG. 1B, LPS triggering of immature DC strongly induced IL-12 (p40/p70) secretion. Combined treatment with DEX and LPS resulted in a strongly reduced (7-fold) IL-12 production compared to LPS treatment alone, whereas DEX treatment only also resulted in a dramatically reduced IL-12 production.

Impaired stimulating capacity of alternatively activated DC

The reduced IL-12 production by DEX treated LPS triggered DC (DEX-LPS) prompted us to assess the T cell stimulatory capacity of these DC. As shown in FIG. 2A, proliferation of BALB/c ($H\text{-}2^d$) splenocytes in a primary MLR response to stimulation with B6 derived ($H\text{-}2^b$) DEX-LPS DC was strongly reduced (and similar to the allogeneic response to untreated immature B6 DC). Similar striking differences of the allogeneic (major and minor histocompatibility antigens mismatched) response were observed when IFN-γ production of the BALB/c splenocytes in response to the various DC used as stimulator cells was measured (FIG. 2B). Besides DEX-LPS DC also the DEX treated immature DC (DEX) induced strongly reduced alloreactive responses as measured by IFN-γ production. Therefore, these results show that mature DC pretreated with DEX have an impaired stimulating capacity.

In vivo reactivity induced by classical versus "alternatively activated" DC

To study the modulation of allo-specific immunity of these DC *in vivo*, these *in vitro* pretreated cells were injected via 2 different routes, either iv or sc. Spleen cells were harvested at different times after injection and restimulated with allogeneic splenocytes *in vitro*. Spleen

cells from mice injected iv with mature DC (LPS), exhibited a high proliferative allogeneic response which was significantly higher than that of untreated control mice or of mice treated with immature DC, DEX treated immature DC (DEX) or DEX-LPS DC (FIG. 3A). Analysis of the production of IFN-γ by the splenocytes of mice injected with the different DC revealed that mice injected iv with the DEX DC showed a similar IFN-γ production as the mice injected with DEX-LPS DC. This response was slightly higher compared to untreated control mice but significantly lower compared to mice injected with untreated immature DC or with LPS DC (FIG. 3B). The proliferative responses of spleen cells from mice injected sc with DEX-LPS DC exhibited a low proliferative allogeneic response which was similar to that of untreated controls and to that of DEX DC (FIG. 4A). The allogeneic IFN-γ response after the DEX-LPS DC treatment was slightly higher or comparable to that of untreated controls, but significantly reduced when compared to mature DC treatment, whereas the DEX DC induced a response similar to mice injected with untreated immature DC (FIG. 4B). The number of IFN-γ producing cells as measured by ELISPOT analysis was 4 times lower after treatment with DEX-LPS DC than after treatment with the DEX DC but comparable to that of untreated controls (data not shown). When the splenocytes were *in vitro* stimulated with C57BL/6 alloantigens for 6 days and restimulated with either conA or C57BL/6 splenocytes, the ELISPOT analysis showed an increase in the number of IL-10 producing cells when compared to untreated or to LPS-DC treated mice (FIG. 4C).

The third-party reactivity was not altered in the DC treated mice compared to untreated mice, indicating that the treatment with alternatively activated H-2^b DC was specific for the H-2^b alloantigens. These experiments demonstrate that DEX-LPS DC induce an alloimmune response, which, based on the *in vitro* parameters tested, showed both quantitative and qualitative differences compared to the alloimmune response found after injection with mature DCs.

Prolonged skin allograft survival after injection with alternatively activated DC

Subsequently, we analyzed the *in vivo* "modulatory" potential of the DEX-LPS DC in a fully allogeneic skin graft model. BALB/c mice were injected sc with either LPS DC or DEX-LPS DC or left untreated. One week after treatment these mice were transplanted with a skin

graft derived from the tail of a donor C57BL/6 mouse. The skins derived from C57BL/6 mice were rejected by the mice injected with LPS DC with a median survival time of 14 days which is not significantly different from the survival in untreated mice (MST 16 days, FIG. 5A). However, when mice were transplanted after injection with DEX-LPS DC, a significantly prolonged allograft survival was found (MST 34 days p=0.039). A similar significant prolongation was observed in 2 other independent experiments using BALB/c mice as responding strain (p=0.023 and p=0.009) and in another study using BM1 mice as responder strain (p=0.008, data not shown).

The prolonged skin graft survival after treatment with alternatively activated H-2^b DC was specific for the H-2^b alloantigens as mice injected with DEX-LPS DC rejected skin grafts from DBA/1 mice (H-2^q) in the same time (MST 14 days, FIG. 5B) as control mice (MST 14 days, untreated or LPS DC treated mice p=0.90, p=0.92 resp). These results show that the DEX-LPS DC are capable of inducing a specific prolongation of complete MHC incompatible skin allograft survival.

DISCUSSION

The present study shows that addition of a glucocorticoid hormone to immature DC results in a decreased proliferative response and a decrease in IFN-γ production by BALB/c splenocytes stimulated by these DCs. In addition we demonstrate that *in vivo* treatment with DEX pretreated mature DC decreased the allogeneic Th1 response as shown by a reduced IFN-γ production *in vitro* and a reduction in number of IFN-γ producing effector cells when the response was compared to mice pretreated with mature DC. This was the case both after sc or iv injection of the DEX pretreated DCs, but even more after *in vivo* treatment with the alternatively activated (DEX-LPS) DC. Pretreatment of recipients with these DC leads to a significantly prolonged skin graft survival.

In conclusion, our studies confirm and extend the practical use of alternatively activated DC for modulation of the alloimmune response and show that these can induce a prolonged skin graft survival even in a complete MHC incompatible donor-recipient combination.

I hereby declare that all statements made herein of my own knowledge are true and that

all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the U.S. Code and that such willful false statements may jeopardize the validity of the patent.

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GOODMAN and GILMAN's The Pharmacological Basis of Therapeutics

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In this textbook, reference to proprietary names of drugs is ordinarily made only in chapter sections dealing with preparations. Such names are given in SMALL-CAP TYPE, usually immediately following the official or nonproprietary titles. Proprietary names of drugs also appear in the Index.

CHAPTER

63 ADRENOCORTICOTROPIC HORMONE; ADRENOCORTICAL STEROIDS AND THEIR SYNTHETIC ANALOGS; INHIBITORS OF ADRENOCORTICAL STEROID BIOSYNTHESIS

Robert C. Haynes, Jr., and Ferid Murad

Adrenocorticotropic hormone (ACTH, corticotropin) and the steroids of the adrenal cortex are considered together in this chapter because the primary physiological and pharmacological effects of ACTH result from the secretion of adrenocortical steroids. Biologically active synthetic analogs of the adrenocorticosteroids are also included, as are substances that alter the pattern of secretion of the adrenal cortex by inhibiting certain biosynthetic reactions. Synthetic steroids and other compounds that inhibit the action of aldosterone on the renal tubule are discussed in Chapter 36.

History. The physiological significance of the adrenals began to be appreciated as a consequence of the description by Addison (1855) of the clinical syndrome resulting from destructive disease of the adrenal glands. His observations interested the physiologist Brown-Séquard (1856), who did the pioneer experiments on the effects of adrenalectomy and concluded that the adrenal glands are essential to life.

By the third decade of this century it was generally recognized that the cortex rather than the medulla is the life-maintaining portion of the gland. Soon the literature was replete with descriptions of the numerous physiological abnormalities exhibited by adrenalectomized animals. The complex nature of adrenocortical deficiency was dramatized in the 1930s by the partisan character of research groups oriented to study either the imbalance of electrolytes or the defects in carbohydrate metabolism present in the deficient state. Renal loss of sodium was convincingly demonstrated to be a characteristic of adrenocortical insufficiency by Harrop and associates (1933) as well as by Loeb and coworkers (1933). Equally convincing was the demonstration of a depletion of carbohydrate stores (Cori and Cori, 1927). Furthermore, hypoglycemia could be corrected by adrenocortical extracts (Britton and Silvette, 1931). Glucose and glycogen, formed under the influence of the adrenal cortex during fasting, appeared to be derived from tissue protein (Long *et al.*, 1940). From these studies there emerged the concepts of two types of adrenocortical hormones. The mineralocorticoids primarily regulate electrolyte homeostasis, and the glucocorticoids are hormones concerned with carbohydrate metabolism. This concept of the dichotomy of "salt" and "sugar" hormones (mineralocorticoids and glucocorticoids) has proven useful and survives at the present time in a modified form.

In 1932, the neurosurgeon Cushing described the syndrome of hypercorticism, which bears his name (Cushing, 1932). The cases Cushing described were those of "pituitary basophilism," recognized subsequently as being a condition characterized by hypersecretion of ACTH. The symptom complex is now known to result from excessive plasma concentrations of adrenocortical hormones, regardless of whether they originate endogenously or as the consequence of therapeutic intervention.

The preparation of adrenocortical extracts with a reasonable degree of activity was first accomplished in 1930 by Swingle and Pfiffner and by Hartman and associates. The existence of biologically active tissue extracts presented a challenge to organic chemists, who by 1942 had isolated, crystallized, and elucidated the structures of 28 steroids from the adrenal cortex (Reichstein and Shoppee, 1943). Five of these compounds—cortisol (hydrocortisone), cortisone, corticosterone, 11-dehydrocorticosterone, and 11-desoxycorticosterone—were demonstrated to be biologically active. Another decade passed before the principal mineralocorticoid was discovered. Deming and Luetscher (1950) found that extracts of urine from patients with edema induced sodium retention and potassium excretion in adrenalectomized rats. The definitive evidence for the source of the active material was provided by Tait and coworkers (1952), who purified the compound with this activity from adrenocortical extracts. The substance was crystallized, the structure was established, and the hormone was eventually named *aldosterone* (Simpson *et al.*, 1954).

Meanwhile, other investigators had turned their attention to the adenohypophysis. The classical studies of Foster and Smith (1926) established the fact that hypophysectomy results in atrophy of the adrenal cortex. By 1933, it had been demonstrated

that cell-free extracts of the anterior pituitary had a stimulating effect upon the adrenal cortex of the hypophysectomized animal. Further chemical fractionation of such extracts led to the isolation of a hormone, ACTH, that acted selectively to cause chemical and morphological changes in the adrenal cortex (Li *et al.*, 1943; Sayers *et al.*, 1943; Astwood *et al.*, 1952). The structure of ACTH was established by Bell and coworkers (1956). Within a few years biologically active peptides were synthesized (Hofmann *et al.*, 1961), as was an ACTH of 39 amino acid residues (Schwyzer and Sieber, 1963). The rate of release of ACTH from the adenohypophysis was shown to be determined by the balance of inhibitory effects of the hormones of the adrenal cortex (Ingle *et al.*, 1938) and the excitatory effects of the nervous system. The hypothalamus was established as the "final common path" for the variety of stimuli impinging on the adenohypophysis.

A detailed analysis of the morphology of the adrenal cortex had suggested to Swann (1940) and to Deane and Greep (1946) that the zona glomerulosa of the adrenal cortex functions relatively independently of the pituitary. Following hypophysectomy, the zona glomerulosa thickens, whereas the fasciculata shrinks markedly and the reticularis disappears almost entirely. These morphological observations, together with the fact that the hypophysectomized rat, in contrast to the adrenalectomized animal, can survive without salt therapy, prompted Swann as well as Deane and Greep to assign to the zona glomerulosa the specific function of autonomously elaborating a hormone regulating electrolyte balance. This hormone is now known to be aldosterone. Subsequent experimental studies have shown that the rate of secretion of aldosterone is regulated by a complex system, of which the pituitary is but one element.

In 1949, Hench and coworkers announced the dramatic effects of cortisone and ACTH in the treatment of rheumatoid arthritis. As early as 1929, Hench was impressed by the fact that arthritic patients, when pregnant or jaundiced, experienced a temporary remission; he believed that a metabolite was responsible for the remission. The possibility that the antirheumatic substance might be an adrenocortical hormone was entertained, and as soon as cortisone was available in sufficient quantity it was tested in a case of acute rheumatoid arthritis. Fortunately, an adequate dose was employed and the response was dramatic. Thereafter, the salutary effects of ACTH were also demonstrated. The observations (Hench *et al.*, 1949) immediately evoked wide interest. Soon, therapeutic applications were extended to other diseases, with results to be presented later in this chapter. The impact upon the medical world can be appreciated from the fact that, in the year following the first published report of the efficacy of cortisone in the treatment of rheumatoid arthritis, the Nobel Prize in Medicine was jointly awarded to Kendall and Reichstein, who were responsible for much of the basic chemical research that led to the synthesis of the steroid, and to Hench, whose contribution has just been described.

In addition to a surge of clinical investigation, the therapeutic success of cortisone stimulated a wave of basic research in the 1950s. In that decade knowledge of the biochemistry of adrenal steroid synthesis and metabolism was brought close to its present level. As noted above, aldosterone was discovered; it was established that ACTH controls the reaction of cholesterol side chain scission (Stone and Hechter, 1954) and acts through the intermediacy of adenosine 3',5'-monophosphate (cyclic AMP) (Haynes *et al.*, 1959); most synthetic analogs of cortisol used today were introduced, and practical technics for determination of cortisol became available to the clinician.

Effective clinical use of the corticosteroids has become possible because of their isolation, elucidation of structure, and economical synthesis. Manipulation of structure has yielded a variety of synthetic analogs, some of which represent significant therapeutic gains in terms of the ratio of anti-inflammatory potency to effects on electrolyte metabolism. However, hopes for elimination of toxicity have not been fulfilled. For this reason, it cannot be overemphasized that the corticosteroids, in pharmacological doses, are powerful drugs with slow cumulative toxic effects on many tissues, which may not be apparent until made manifest by a catastrophe.

ADRENOCORTICOTROPIC HORMONE

Chemistry. The structure of human ACTH, a peptide of 39 amino acid residues, is shown in Figure 63–1. Loss of one amino acid from the N-terminal end of the molecule by hydrolytic cleavage results in complete loss of biological activity. In contrast, a number of amino acids may be split off the C-terminal end with no effect on potency. A 20–amino acid peptide (sequence 1 through 20, Figure 63–1) retains the activity of the parent hor-

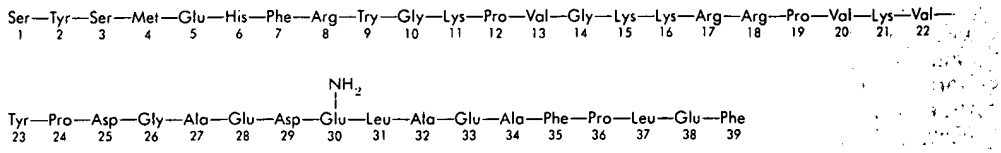


Figure 63–1. *Amino acid sequence of human ACTH.*

Ovine, porcine, and bovine ACTHs differ from human ACTH only at amino acid positions 25, 31, and 33 (Li, 1972).

mone. The structure-activity relationship of ACTH has been reviewed by Otsuka and Inouye (1975). The structural relationships between ACTH, endorphins, lipotropins, and the melanocyte-stimulating hormones are discussed in Chapter 59.

Actions on Adrenal Cortex. ACTH stimulates the human adrenal cortex to secrete cortisol, corticosterone, aldosterone, and a number of weakly androgenic substances. In the absence of the adenohypophysis, the adrenal cortex undergoes atrophy and the rates of secretion of cortisol and corticosterone, which are markedly reduced, do not respond to otherwise-effective stimuli. Although ACTH can stimulate secretion of aldosterone, the rate of secretion is relatively independent of the adenohypophysis, and this explains the nearly normal electrolyte balance in the hypophysectomized animal. The zona glomerulosa is the least affected by atrophic changes that follow hypophysectomy, and it is the glomerulosa that is mainly responsible for the elaboration of aldosterone.

Prolonged administration of large doses of ACTH induces hyperplasia and hypertrophy of the adrenal cortex with continuous high output of cortisol, corticosterone, and androgens.

Mechanism of Action. ACTH acts to stimulate the *synthesis* of adrenocortical hormones; if it facilitates the release of preformed steroids from the adrenal cortex at all, this effect is overshadowed by the greater effect on synthesis. ACTH, as many other hormones, controls its target tissue through the agency of cyclic AMP. Thus, treatment with ACTH causes an increase in concentration of the cyclic nucleotide within adrenocortical cells (Haynes, 1958); cyclic AMP mimics ACTH in stimulating steroidogenesis (Haynes *et al.*, 1959) and in maintaining the weight of the adrenal after hypophysectomy (Ney, 1969). ACTH reacts with a specific hormone receptor in the adrenal-cell plasma membrane, and the result is a stimulation of adenylate cyclase activity and the formation of cyclic AMP.

The principal metabolic site at which steroidogenesis is regulated by the cyclic nucleotide is the oxidative cleavage of the side chain of cholesterol, the reaction that results in the formation of pregnenolone (*see* Figure 63–3, page 1465). This step is rate limiting in the sequence of reactions that leads to the formation of adrenal steroid hormones (Stone and Hechter, 1954). Exposure of adrenocortical cells to ACTH together with aminoglutethimide (to block side chain cleavage; *see* below) leads to increased amounts of cholesterol within the adrenal mitochondria, the locus of the side chain–cleaving enzyme (Mahaffee *et al.*, 1974). Furthermore, cholesterol bound to cytochrome P-450 is increased by ACTH (Bell and Harding, 1974; Paul *et al.*, 1976). These findings, together with evidence that the availability of cholesterol is the factor that limits the rate of the cleavage reaction in intact mitochondria (Kahnt *et al.*, 1974), suggest that ACTH, via cyclic AMP, stimulates the initial reaction in steroidogenesis from cholesterol by making the substrate available in increased concentration to the enzyme within the mitochondria. ACTH stimulates the formation of free cholesterol in the gland by activating cholesterol esterase, and this activation is apparently accomplished by phosphorylation of the enzyme (Beckett and Boyd, 1975; Pittman and Steinberg, 1977). ACTH also acts to increase the availability of cholesterol by stimulating its uptake from plasma lipoproteins (Gwynne *et al.*, 1976).

The trophic effects of ACTH on the adrenal cortex are little understood beyond the fact that they, like stimulation of steroidogenesis, appear to be mediated by cyclic AMP (Ney, 1969). The regulation of the adrenal cortex by ACTH has been reviewed by Kimura (1981).

Extra-adrenal Effects of ACTH. Large doses of ACTH given to adrenalectomized animals cause a number of metabolic changes, including ketosis, lipolysis, hypoglycemia (early after administration), and resistance to insulin (late after administration). These extra-adrenal effects are of doubtful physiological significance, particularly since large doses are needed to induce them (Engel, 1961). Intravenous administration of ACTH (synthetic or porcine, but not bovine) leads to a transient elevation of the concentration of growth hormone in the plasma of adults but not children (Lee *et al.*, 1973).

Natural and synthetic corticotropins darken the isolated skin of the frog; this is not surprising since the amino acid sequence, 1 through 13, is identical with that of the melanocyte-stimulating hormone, α-MSH. Large doses of highly purified α-MSH and ACTH have been demonstrated to darken the skin of adrenalectomized human subjects. The hyperpigmentation of the skin that occurs in Addison's disease is thought to result from the high concentrations of ACTH that circulate in this condition (Thody, 1977; *see* Chapter 59).

Regulation of the Secretion of ACTH. The fluctuations in the rates of secretion of cortisol, corticosterone, and, to some extent, aldosterone are determined by the fluctuations in the release of ACTH from the adenohypophysis. The adenohypophysis, in turn, is under the influence of the *nervous system* and *negative-feedback control* exerted by *corticosteroids* (*see* Gann *et al.*, 1981).

Nervous System: The Final Common Path. Stimuli that induce release of ACTH travel by neural paths converging on the median eminence of the

hypothalamus. The functional link between the median eminence and the adenohypophysis, the final common path, is vascular, not neural. In response to an appropriate stimulus, corticotropin-releasing factor (CRF) is elaborated at neuronal endings in the median eminence and transported in the hypophyseal-portal vessels to the adenohypophysis, where it stimulates the secretion of ACTH. The isolation and synthesis of an ovine CRF were reported by Vale and coworkers (1981). This polypeptide, which contains 41 amino acid residues, increases the concentrations of ACTH and cortisol in plasma when given intravenously to man. It does not change the concentrations of prolactin, growth hormone, thyrotropin, or the gonadotropins (Grossman *et al.*, 1982). Intravenous injection of 100 μg of CRF causes an exaggerated response in patients with Cushing's syndrome due to pituitary hyperfunction. It is thus useful in determining the cause of the disease and helps to rule out ectopic production of ACTH or functional tumors of the adrenal cortex as responsible (Muller *et al.*, 1983). (For additional information, *see* Conference, 1985.)

ACTH is synthesized in basophilic cells of the adenohypophysis and, like many other peptide hormones, it is derived from a larger precursor; the prohormone is a glycoprotein of about 30,000 molecular weight. As indicated in Figure 59–2 (page 1380), the precursor of ACTH includes the sequences of MSH, the lipotropins, and the endorphins. In man, the role of these three groups of active peptides remains conjectural and a subject of active investigation. The complex processing of the prohormone to ACTH, β-lipotropin, and other peptides has been studied extensively (*see* Loh and Loriaux, 1982).

Negative Feedback of the Corticosteroids (Cortisol and Corticosterone). Administration of certain corticosteroids suppresses the secretion of ACTH, reduces the store of ACTH in the adenohypophysis, and induces morphological changes (hyalinization of the basophilic cells) suggestive of functional impairment of the adenohypophysis. The adrenal cortex itself undergoes atrophy. In contrast, adrenalectomized animals and patients with Addison's disease have abnormally high concentrations of ACTH in the blood even under optimal environmental conditions. When a stimulus is applied to an adrenalectomized animal, the concentration of ACTH reaches even higher levels. These observations point out the important inhibitory role of the corticosteroids and clearly demonstrate that ACTH release remains under control of the nervous system in the absence of corticosteroid feedback. Secretion of ACTH at any instant is determined by the balance of neural excitatory and corticosteroid inhibitory effects.

Mechanism of Feedback by Corticosteroids. Binding of glucocorticoids has been detected in the pituitary, hypothalamus, and other areas of the brain (McEwen, 1979); however, the link between such binding and inhibition of secretion of ACTH has not been established. There is evidence of control at both hypothalamic and hypophyseal sites (*see* Gann *et al.*, 1981). Nakanishi and coworkers (1977) demonstrated that glucocorticoids cause a decrease in the level of mRNA for ACTH in the pituitary, suggesting control may be at least in part at the transcriptional level. It should be noted, however, that glucocorticoids can cause a fall in the plasma concentration of ACTH that is so rapid that other mechanisms may also be utilized (Johnson *et al.*, 1979).

Examples of Effective Stimuli of Secretion. A number of conditions have been demonstrated to stimulate adrenocortical secretion in man. These include the agonal state, severe infections, surgery, parturition, cold, exercise, and emotional stress. Stressful stimuli override the normal negative-feedback control mechanisms, and plasma concentrations of adrenocortical steroids can be elevated within a few minutes of the initiation of an appropriate stimulus.

Diurnal Cycles in Adrenocortical Activity. The rate of secretion of cortisol by the adrenal cortex of a normal human subject under optimal conditions is about 20 mg per day. However, the rate is not steady and exhibits rhythmic fluctuations; concentrations of adrenocortical steroids in plasma are relatively high in the early-morning hours, decline during the day, and reach a minimum about midnight. Plasma concentrations of ACTH are higher at 6 A.M. than at 6 P.M. The diurnal patterns of glucocorticoids and ACTH are not observed in patients with Cushing's disease, and this factor is considered in the diagnosis of the disorder.

Absorption and Fate. ACTH is readily absorbed from parenteral sites, and it is usually administered by intramuscular injection and occasionally by intravenous infusion. The hormone rapidly disappears from the circulation following its intravenous administration; in man, the half-life in plasma is about 15 minutes because of rapid enzymatic hydrolysis.

Bioassay. The USP has adopted the *Third International Standard for Corticotropin* (Bangham *et al.*, 1962) as the reference standard in the United States. Potency is based on an assay in hypophysectomized rats in which depletion of adrenal ascorbic acid is measured after subcutaneous administration of the ACTH. All commercial preparations are now described in these units only.

Preparations, Dosage, and Routes of Administration. *Corticotropin for injection* (ACTH) is available as a lyophilized powder (ACTHAR) for s

cutaneous, intramuscular, or intravenous use. The preparation is derived from the pituitaries of mammals used for food. Maximal adrenocortical secretion is obtained in adults with a total dose of 25 USP units infused intravenously for 8 hours.

Repository corticotropin injection (CORTROPHIN GEL, H.P. ACTHAR GEL) is administered either intramuscularly or subcutaneously. It is a highly purified ACTH in gelatin solution. Typical doses are 40 to 80 units, given every 1 to 3 days. *Corticotropin zinc hydroxide suspension* (CORTROPHIN-ZINC) is a preparation of purified corticotropin adsorbed on zinc hydroxide, intended for intramuscular injection. Again, usual doses are 40 to 80 units every 1 to 3 days.

Cosyntropin (CORTROSYN) is a synthetic peptide corresponding to amino acid residues 1 to 24 of human ACTH. This preparation, approved for diagnostic purposes, is given intramuscularly or intravenously in a dose of 0.25 mg (equivalent to 25 units).

Therapeutic and Diagnostic Applications of ACTH. At the present time, the most important use of ACTH is as a *diagnostic agent* in adrenal insufficiency. For this purpose, ACTH is administered and the concentration of cortisol in plasma is determined. A normal increase in plasma cortisol rules out primary adrenocortical failure. If there is no acute response, prolonged or repeated administration of ACTH may be required. In cases of pituitary insufficiency, prolonged treatment can be expected to elicit a rise in plasma cortisol concentration.

Therapeutic uses of ACTH have included the treatment of adrenocortical insufficiency and nonendocrine disorders that are responsive to glucocorticoids. However, therapy with ACTH is less predictable and much less convenient than is that with appropriate steroids. Furthermore, ACTH stimulates secretion of mineralocorticoids and, therefore, may cause acute retention of salt and water. While this generally does not persist with continuing therapy, it is a potentially serious problem in patients who have cardiac insufficiency. ACTH would obviously be of no value in the treatment of primary adrenocortical failure. Furthermore, there is no substantial evidence that therapeutic goals can be attained with ACTH in secondary adrenocortical insufficiency that cannot be attained with appropriate doses of currently available steroids. It must be kept in mind, however, that ACTH and corticosteroids are not pharmacologically equivalent. Treatment with ACTH exposes the tissues to a mixture of glucocorticoids, mineralocorticoids, and androgens, in contrast to the conventional, contemporary practice of administering a single glucocorticoid. It is possible that the steroid mixture resulting from adrenal stimulation by ACTH has effects that differ significantly from those of a single, synthetic glucocorticoid. Thus, Grahame (1969) reported the absence of dermal atrophy in patients treated for prolonged periods of time with ACTH, in contrast to that found with corticosteroid treatment. This has been tentatively attributed to a protective action of androgens against the inhibitory effects of glucocorticoids on fibroblasts (Harvey and Grahame, 1973).

Clinical Toxicity of ACTH. The toxicity of ACTH, aside from rare hypersensitivity reactions, is entirely attributable to the increased rate of secretion of adrenocorticosteroids (*see* below). Hypersensitivity reactions, ranging from mild fever to anaphylaxis and death, have been reported. The synthetic ACTH peptides are thought to be less antigenic than is the parent molecule. Nevertheless, hypersensitivity to them does occur (Forssman and Mulder, 1973). Because it stimulates synthesis and secretion of mineralocorticoids and androgens, ACTH causes more sodium retention, a greater degree of hypokalemic alkalosis, and more acne than do the synthetic congeners of cortisol.

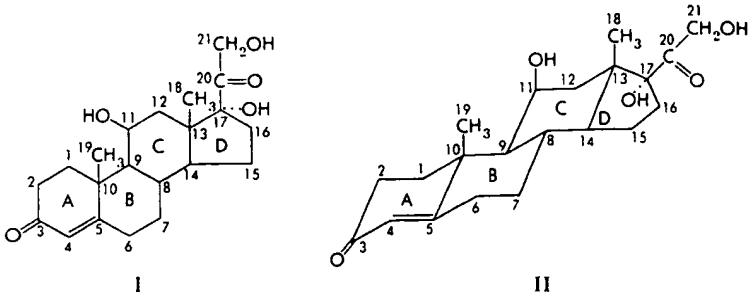
ADRENOCORTICAL STEROIDS

The adrenal cortex synthesizes two classes of steroids: the corticosteroids (glucocorticoids and mineralocorticoids) with 21 carbon atoms and the androgens with 19. A typical corticosteroid, *cortisol*, is shown in Figure 63–2; typical androgens are shown in Figure 63–3.

Adrenocorticosteroid Biosynthesis. Cholesterol is an obligatory intermediate in the biosynthesis of corticosteroids. Although the adrenal cortex synthesizes cholesterol from acetate by processes similar to those in liver, the greater part of the cholesterol (60 to 80%) utilized for corticosteroidogenesis comes from exogenous sources, both at rest and following administration of ACTH. Adrenocortical cells thus have large numbers of receptors that mediate the uptake of low-density lipoprotein, the predominant source of cholesterol (*see* Chapter 34). Cholesterol is enzymatically converted to 21-carbon corticosteroids and 19-carbon weak androgens by a series of steps presented in simplified form in Figure 63–3. Most of the reactions are catalyzed by mixed-function oxidases that contain cytochrome P-450 and require NADPH and molecular oxygen.

In addition to other androgens, the adrenal cortex secretes testosterone; however, about half the plasma testosterone of normal women is derived from androstenedione at an extra-adrenal site.

Adrenocorticosteroids are not stored in the adrenal. The amounts of corticosteroids found in adrenal tissue are insufficient to maintain normal rates of secretion for more than a few minutes in the absence of continuing biosynthesis. For this reason, the rate of biosynthesis is tantamount to the rate of secretion. Table 63–1 shows typical rates of secretion of the physiologically most important corticosteroids in man—cortisol and aldosterone—and also their approximate concentrations in peripheral plasma. The mechanism of control of steroidogenesis by ACTH has been discussed above, and the regulation of aldosterone synthesis by renin and angiotensin is described in Chapter 27.



I II

Figure 63–2. *Structure, stereochemistry, and nomenclature of adrenocorticosteroids, as typified by cortisol (hydrocortisone).*

The four rings—A, B, C, and D—are not in a flat plane, as conventionally represented in *I*, but have the approximate configuration shown in *II*. (The planarity of the valence angles about the double bond between C 4 and C 5 prevents the chair form of ring A, as shown, from being an energetically probable conformational state. As a result, ring A is in a half-chair conformation, not easily represented in two dimensions.) Orientation of the groups attached to the steroid ring system is importantly related to biological activity. The methyl groups at C 18 and C 19, the hydroxyl group at C 11, and the two-carbon ketol side chain at C 17 project above the plane of the steroid and are designated β. Their connection to the ring system is shown by full-line bonds. The hydroxy at C 17 projects below the plane and is designated α, and the connection to the ring is shown by a dotted bond. The ketone at C 3 in association with the double bond between C 4 and C 5 in ring A is an important structural feature of the biologically active corticosteroids. Reduction of the ketone at C 3 leads to the formation of two isomers: one, 3β-hydroxy; the other, 3α-hydroxy. Saturation of the 4,5 double bond leads to the formation of two isomers: 5α and 5β. Reduction of the ketone at C 20 creates an asymmetrical carbon at this site, the two possible isomers being designated α and β.

In formal chemical nomenclature, the adrenocortical hormones are described as derivatives of androstane or of pregnane. Double bonds are indicated by the symbol Δ with superscripts to indicate the position of the double bond. In this convention, cortisol is designated 11β,-17α,21-trihydroxy-Δ^4-pregnene-3,20-dione. Dehydroepiandrosterone is designated 3β-hydroxy-Δ^5-androsten-17-one.

PHYSIOLOGICAL FUNCTIONS AND PHARMACOLOGICAL EFFECTS

The effects of the corticosteroids are numerous and widespread. They influence carbohydrate, protein, and lipid metabolism; electrolyte and water balance; and the functions of the cardiovascular system, the kidney, skeletal muscle, the nervous system, and other organs and tissues. Furthermore, the corticosteroids endow the organism with the capacity to resist many types of noxious stimuli and environmental change. The adrenal cortex is the organ *par excellence*, of homeostasis, being responsible to a large extent for the relative freedom that higher organisms exhibit in a constantly changing environment. In the absence of the adrenal cortex, survival is possible but only under the most rigidly prescribed conditions; for example, food must be available regularly, sodium chloride ingested in relatively large quantities, and environmental temperature maintained within a suitably narrow range.

A given dose of corticosteroid may be *physiological* or *pharmacological*, depending on the environment and the activities of the organism. Under favorable conditions, a small dose of corticosteroid maintains the adrenalectomized animal in a state of well being. Under adverse conditions a relatively large dose is needed if the animal is to survive. This same large dose given repetitively under optimal conditions induces

Table 63–1. RATES OF SECRETION AND TYPICAL PLASMA CONCENTRATIONS OF THE MAJOR BIOLOGICALLY ACTIVE CORTICOSTEROIDS IN MAN

		CORTISOL	ALDOSTERONE
Rate of secretion under optimal conditions, mg/day		20	0.125
Concentrations in peripheral plasma of man, μg/dl	8 A.M.	16	0.01
	4 P.M.	4	

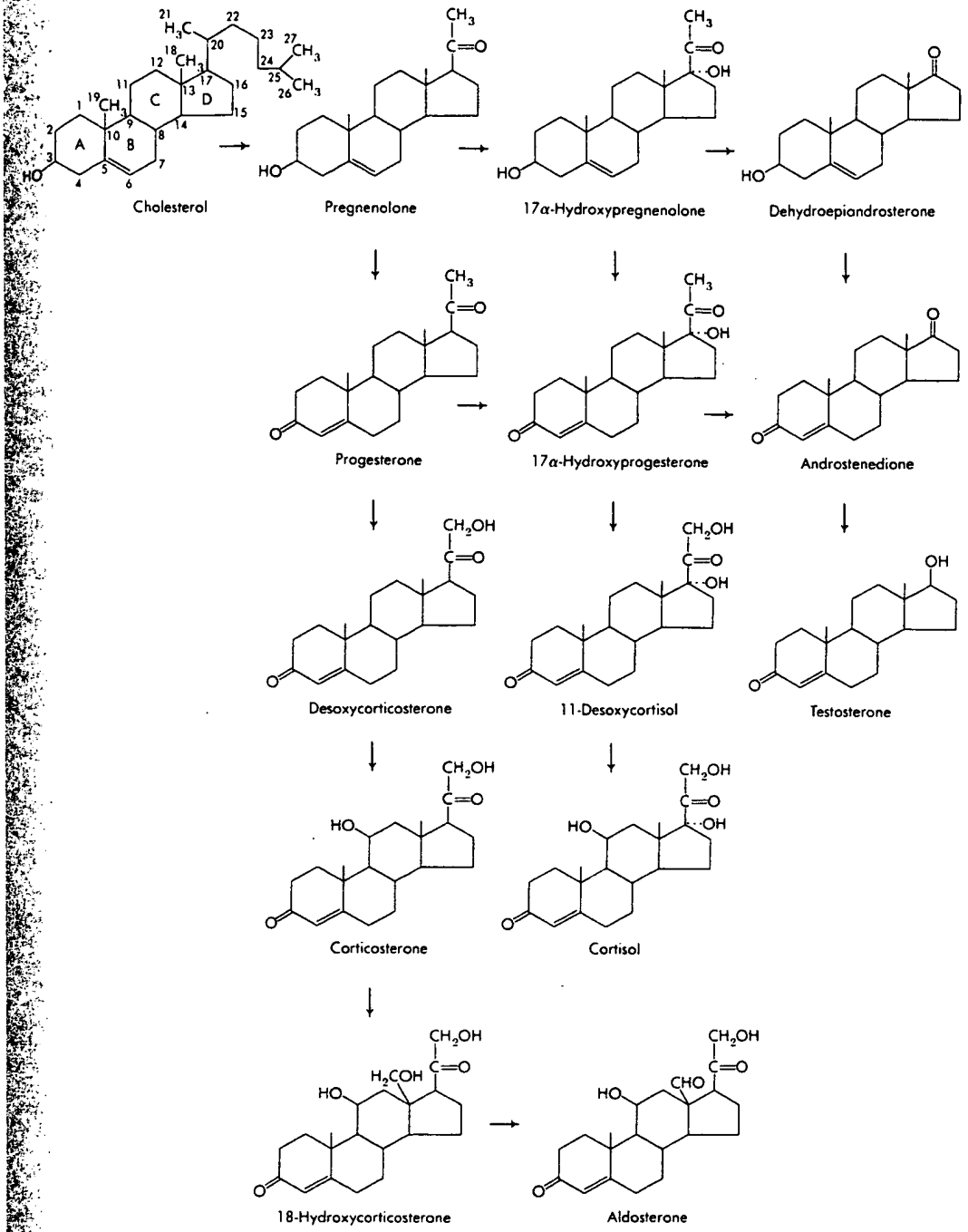


Figure 63–3. *Principal pathways for biosynthesis of adrenocorticosteroids and adrenal androgens.*

hypercorticism, that is, signs of excess of corticosteroid. The fluctuations in the secretory activity of a normal subject are presumed to reflect the varying needs of the organism for corticosteroids.

The actions of corticosteroids are often complexly related to the functions of other hormones. For example, in the absence of lipolytic hormones, cortisol even in large concentrations has virtually no effect on

the rate of lipolysis in adipose tissue *in vitro*. Likewise, a sympathomimetic amine has only slight effect on the rate of lipolysis if there is a deficiency of glucocorticoids. However, if a necessary minimal amount of cortisol is added, the lipolytic effect of the sympathomimetic amine becomes evident. The necessary but not sufficient role of corticosteroids acting in concert with other regulatory forces has been termed "permissive" by Ingle (1954).

Certain of the biological actions of the corticosteroids lend themselves to quantitative measurement. Estimates of the potencies of naturally occurring and synthetic corticosteroids in the categories of *sodium retention* (reduction of sodium excretion by the kidney of the adrenalectomized animal), *hepatic deposition of glycogen*, and *anti-inflammatory effect* (inhibition of the action of an agent that induces inflammation) are presented in Table 63–2. It should be noted that such values are not fixed ratios but vary considerably with the conditions of the bioassays used. Potencies of steroids as judged by ability to sustain life in the adrenalectomized animal closely parallel those determined for sodium retention. Potencies based on liver glycogen deposition, anti-inflammatory effect, work capacity of skeletal muscle, and involution of lymphoid tissue closely parallel one another. Dissociations exist between potencies based on sodium retention and on liver glycogen deposition; traditionally the corticosteroids have thus been classified into *mineralocorticoids* and *glucocorticoids*, according to potencies in the two categories. Desoxycorticosterone, the prototype of the mineralocorticoids, is highly potent in regard to sodium retention but without effect on hepatic glycogen deposition. Cortisol, the prototype of the glucocorticoids, is highly potent in regard to liver glycogen deposition but weak in regard to sodium retention. The naturally occurring corticosteroids cortisol and cortisone as well as synthetic corticosteroids such as prednisolone and triamcinolone are classified as glucocorticoids. However, corticosterone is a steroid that has modest but significant activities in both categories. In contrast, aldosterone is exceedingly potent with respect to sodium retention, with modest potency for liver glycogen deposition. At rates secreted by the adrenal cortex or in doses that exert maximal effects on electrolyte balance, aldosterone has no significant effect on carbohydrate metabolism; it is thus classified as a mineralocorticoid.

Table 63–2. RELATIVE POTENCIES OF CORTICOSTEROIDS

	SODIUM RETENTION	LIVER GLYCOGEN DEPOSITION	ANTI-INFLAMMATORY EFFECT
Natural Steroids			
Cortisol	1 *	1	1
Cortisone	0.8 *	0.8	0.8
Corticosterone	15	0.35	0.3
11-Desoxycorticosterone	100	0	0
Aldosterone	3000	0.3	?
Synthetic Steroids			
Prednisolone	<1 *	4	4
Triamcinolone	0	5	5

* Promotes sodium excretion under certain circumstances.

In the following descriptions of the physiological functions and the pharmacological effects of the corticosteroids, the terms *mineralocorticoid* and *glucocorticoid* will be employed for convenience. It is to be emphasized that the biological characteristics of the corticosteroids range over a spectrum from that of a strictly mineralocorticoid type at the one end to that of a strictly glucocorticoid type at the other. A comprehensive review of the actions of the glucocorticoids is that edited by Baxter and Rousseau (1979).

Mechanism of Action. Corticosteroids, like other steroid hormones, are thought to act by controlling the rate of synthesis of proteins. As is true with estrogens (Chapter 61), the corticosteroids react with receptor proteins in the cytoplasm of sensitive cells to form a steroid-receptor complex. Such receptors have been identified in many tissues (Ballard *et al.*, 1974). The steroid-receptor complex undergoes a modification, as noted by an increase in the sedimentation constant; following this, the complex moves into the nucleus, where it binds to chromatin. Information carried by the steroid or more likely by the receptor protein directs the genetic apparatus to transcribe RNA. This is established by demonstrations that glucocorticoids, in appropriate tissues, increase the quantity of mRNA that codes for enzymes whose synthesis is stimulated by these hormones (Schutz *et al.*, 1975; Iynedjian and Hanson, 1977). A more precise understanding of the mechanism by which glucocorticoids activate transcription of specific mRNA

may soon be forthcoming. Glucocorticoids specifically stimulate the rate of viral gene transcription in cultured tumor cells that bear murine mammary tumor virus, and this provides an excellent model system for study of the action of these hormones. The steroid-receptor complex binds *in vitro* to specific sequences of the viral DNA (Payvar *et al.*, 1981). The segments of DNA that are recognized are the long terminal repeat sequences that are known to be sites where transcription is initiated (Govindan *et al.*, 1982).

Steroid hormones thus stimulate transcription and ultimately the synthesis of specific proteins. While this is true for corticosteroids in some tissues, such as the liver, in other tissues, for example, lymphoid cells, the overall effect of the hormones is a catabolic one. This suggests that the steroid-receptor complex may inhibit rather than stimulate transcription in these instances. However, Makman and coworkers (1971) presented evidence suggesting that steroids act in lymphatic cells to stimulate the synthesis of an inhibitory protein, which presumably causes the catabolic effects.

Carbohydrate and Protein Metabolism. The effects of adrenocortical hormones on carbohydrate and protein metabolism are epitomized in the teleological view that these steroids have evolved to protect glucose-dependent cerebral functions by stimulating the formation of glucose, diminishing its peripheral utilization, and promoting its storage as glycogen. Adrenalectomized animals exhibit no marked abnormality in carbohydrate metabolism if food is regularly available. Under such circumstances, normal concentrations of glucose in the plasma are maintained and glycogen is stored in the liver. However, a brief period of starvation rapidly depletes carbohydrate reserves. The concentration of glycogen in the liver, and to a lesser extent that in muscle, decreases and hypoglycemia develops. In light of these facts, it is not surprising that the adrenalectomized animal is hypersensitive to insulin. Patients with Addison's disease have similar abnormalities in carbohydrate metabolism.

Administration of a glucocorticoid such as cortisol corrects the defect in carbohydrate metabolism of the adrenalectomized animal; glycogen stores, particularly in the liver, are increased; concentrations of glucose in plasma remain normal during fasting; sensitivity to insulin returns to normal. Increased excretion of nitrogen accompanies the increased production of glucose, indicating that protein is converted to carbohydrate (Long *et al.*, 1940). Prolonged exposure to large doses of glucocorticoids leads to an exaggeration of these changes in glucose metabolism, so that a diabetic-like state is produced: glucose in the plasma tends to be elevated in the fasting subject, there is increased resistance to insulin, glucose tolerance is decreased, and glucosuria may be present.

The mechanism by which the glucocorticoids inhibit utilization of glucose in peripheral tissues is not understood. Decreased uptake of glucose has been demonstrated in adipose tissue, skin, fibroblasts, and thymocytes as a result of glucocorticoid action.

Glucocorticoids promote gluconeogenesis by both peripheral and hepatic actions. Peripherally these steroids act to mobilize amino acids from a number of tissues. This catabolic action of the glucocorticoids is reflected in the atrophy of lymphatic tissues, reduced mass of muscle, osteoporosis (reduction in protein matrix of bone followed by calcium loss), thinning of the skin, and a negative nitrogen balance. Amino acids funnel into the liver, where they serve as substrates for enzymes involved in the production of glucose and glycogen.

In the liver the glucocorticoids induce *de-novo* synthesis of a number of enzymes involved in gluconeogenesis and amino acid metabolism. For example, the hepatic enzymes phosphoenolpyruvate carboxykinase, fructose-1,6-diphosphatase, and glucose-6-phosphatase, which catalyze reactions of glucose synthesis, are increased in concentration. However, induction of these enzymes requires a matter of hours and cannot account for the earliest effects of the hormones on gluconeogenesis. More rapid effects of glucocorticoids are apparent on hepatic mitochondria, such that they carboxylate pyruvate to form oxaloacetate at an accelerated rate (Adam and Haynes, 1969). This is the first reaction in the synthesis of glucose from pyruvate.

Prolonged, but not acute, treatment with glucocorticoids has been found to elevate the concentration of glucagon in the plasma (Marco *et al.*, 1973; Wise *et al.*, 1973). Inasmuch as glucagon itself stimulates gluconeogenesis, the rise in glucagon should also contribute to the enhanced synthesis of glucose. The deposition of glycogen in the liver found after treatment with glucocorticoids is thought to be the consequence of activation of hepatic glycogen synthase. This activation requires the presence of insulin but is not mediated by a rise in the concentration of insulin (Vanstapel *et al.*, 1982).

Lipid Metabolism. Two effects of corticosteroids on lipid metabolism are firmly established. The first is the dramatic redistribution of body fat that occurs in the hypercorticoid state. The other is the facilitation of the effect of adipokinetic agents in eliciting lipolysis of the triglycerides of adi-

pose tissue. A number of other effects of corticosteroids on lipids have been reported, but in few, if any, instances have they turned out to be direct actions of the corticosteroids themselves.

Administration of large doses of glucocorticoids to human subjects over a long period of time or the hypersecretion of cortisol that occurs in Cushing's syndrome leads to a peculiar alteration in fat distribution. There is a gain of fat in depots in the back of the neck ("buffalo hump"), supraclavicular area, and face ("moon face") and a loss of fat from the extremities. One hypothesis to explain this phenomenon is that of Fain and Czech (1975), who proposed that the adipose tissue that hypertrophies in Cushing's syndrome responds preferentially to the lipogenic and antilipolytic actions of the elevated concentrations of insulin evoked by glucocorticoid-induced hyperglycemia. According to this hypothesis, adipocytes in the extremities, in contrast to those of the trunk, are less sensitive to insulin and more sensitive to the glucocorticoid-facilitated lipolytic effects of other hormones.

The mobilization of fat from peripheral fat depots by epinephrine, norepinephrine, or adipokinetic peptides of the adenohypophysis is markedly blunted in the absence of the adrenal cortex or the adenohypophysis. Cortisol acts in adipose tissue to facilitate the lipolytic response to cyclic AMP, rather than to enhance its accumulation. Hypophysectomy in rats has only a slight effect on the accumulation of cyclic AMP after exposure of adipose tissue to graded doses of epinephrine (Birnbaum and Goodman, 1973); however, hypophysectomy greatly decreases the lipolytic response of adipose tissue to the cyclic nucleotide. Treatment with cortisol restores the normal response to lipolytic hormones and to cyclic AMP (Goodman, 1968). Plasma lipids are not changed consistently in either hypocorticism or hypercorticism.

Electrolyte and Water Balance. Mineralocorticoids act on the distal tubules of the kidney to enhance the reabsorption of sodium ions from the tubular fluid into the plasma; they increase the urinary excretion of both potassium and hydrogen ions. The consequences of these three primary effects in concert with similar actions on cation transport in other tissues appear to account for the entire spectrum of physiological and pharmacological activities that are characteristic of the mineralocorticoids. Thus, the primary features of *hypercorticism* are positive sodium balance and expansion of the extracellular fluid volume, normal or slight increase in the concentration of sodium in the plasma, hypokalemia, and alkalosis. In contrast, those of the deficient state, *hypocorticism*, are sodium loss, hyponatremia, hyperkalemia, contraction of the extracellular fluid volume, and cellular hydration. A defect of major consequence in adrenocortical insufficiency is the renal loss of sodium. The renal tubules normally reabsorb practically all the sodium filtered at the glomerulus. For example, on an ordinary diet, 99.5% may be reabsorbed to maintain sodium balance. Typically, in a patient with Addison's disease under the same circumstances of dietary intake, maximal reabsorption attainable is 98.5%. Since approximately 24,000 mEq of sodium is filtered per day, the 1% difference between reabsorption in the normal subject and reabsorption in the patient with Addison's disease amounts to a loss of 240 mEq of sodium per day. The gravity of the situation is obvious when one considers that this amount of sodium is normally present in 1.7 liters of extracellular fluid. Proportionately more sodium than water is lost through the kidney and the concentration of extracellular sodium decreases; extracellular fluid becomes hypoosmotic, and water shifts from the extracellular into the intracellular compartment. This shift, together with the renal loss of water, results in a marked reduction in the volume of the extracellular fluid. Cells are hydrated, and the increase in the hematocrit value is due not only to a shrinkage of the plasma volume but also to the swelling of the erythrocytes. Hyperkalemia and the tendency toward acid-base disturbances are a result of impairments in the excretion of potassium and of hydrogen ions. Without administration of mineralocorticoids or sodium chloride solution or both, a rapid downhill course ensues in adrenocortical insufficiency. The shrinkage of extracellular fluid volume, the cellular hydration, and the hypodynamic state of the cardiovascular system combine to cause circulatory collapse, renal failure, and death.

In adrenocortical insufficiency, a basic defect in ion transport occurs in a variety of secretory cells. Not only the kidney but also the salivary glands, the sweat glands, the exocrine pancreas, and the mucosa of the gastrointestinal tract elaborate fluids abnormally high in the concentration of sodium and abnormally low in the concentration of potassium. In the patient with Addison's disease, sweating may contribute significantly to the negative balance of sodium.

Aldosterone is by far the most potent of the naturally occurring corticosteroids with regard to electrolyte balance and plays an important role in the regulation of sodium and potassium balance. Evidence of this is the relatively normal electrolyte balance exhibited by the hypophysectomized animal as a result of continued secretion of aldosterone by the adrenal cortex. The increased rate of secretion of aldosterone that occurs in man when dietary salt is severely limited would appear to be a compensatory adjustment of physiological importance. However, *changes* in the rate of secretion of aldosterone are not the cause of *rapid* changes that may occur in sodium excretion. The latent period of action of the steroid is too long.

The intravenous administration of aldosterone to a normal subject is followed, after a delay of about an hour, by a decrease in the rate of renal sodium ion excretion and an increase in the rate of potassium ion and hydrogen ion excretion. If the administration of relatively large amounts of aldosterone is continued over a period of more than 10 to 14 days, sodium excretion again equals sodium intake. However, potassium ion and hydrogen ion excretion continues at an accelerated rate, resulting in hypokalemic hypochloremic alkalosis. The mechanism of "escape" from acute sodium retention is not understood, but it is not due to suppression of the renin-angiotensin system. The effects of the mineralocorticoids have been reviewed by Mulrow and Forman (1972).

The morphological complexity of the mammalian kidney presents a formidable obstacle to an attack on the question of how aldosterone increases sodium reabsorption. Aldosterone stimulates sodium transport by the toad bladder, and it is understandable that investigators have turned to this structurally simple organ as an experimental system.

Studies with the toad bladder have indicated that aldosterone, like other steroids, probably acts to initiate transcription of RNA that serves as template for the synthesis of a protein or proteins. This hypothetical "aldosterone-induced protein" is thought to facilitate the transport of sodium ions from the lumen of the distal tubules through the tubular cells and into the extracellular fluid. The most widely accepted model to describe the action of aldosterone is the following (Marver, 1980). The sodium ions of the tubular filtrate enter the cells of the distal tubules down a concentration gradient through the cell membrane facing the tubular lumen (apical or mucosal surface). Aldosterone and other mineralocorticoids facilitate this diffusion by increasing the permeability of the apical membrane to sodium ions. Sodium ions therefore enter the cells at an accelerated rate and are pumped out into the extracellular space at the serosal surface by a Na^+,K^+-activated adenosine triphosphatase (Na^+,K^+-ATPase).

The mechanisms of the enhanced excretion of potassium and hydrogen ions are less well understood. For practical purposes one may visualize these ions as being "exchanged" for the additional sodium ions reabsorbed under the influence of the steroids, because the sum of the equivalents of the additional potassium and hydrogen ions excreted is equal to that of the additional sodium ions retained.

The *glucocorticoids* decrease the absorption of calcium from the intestine and increase its renal excretion, thus producing a negative balance of the cation. These effects are considered to be the basis of the favorable therapeutic response to glucocorticoids seen in hypercalcemia (*see* Chapter 65).

Desoxycorticosterone is a natural mineralocorticoid of some historical interest for it was the first corticosteroid to be synthesized and made available for the treatment of Addison's disease. Desoxycorticosterone is practically devoid of glucocorticoid effects. Qualitatively, it is identical to aldosterone in its effects on electrolytes; quantitatively, it is about 3% as potent (*see* Table 63–2). Thus, despite the fact that the concentration of desoxycorticosterone in plasma is approximately the same as that of aldosterone, it apparently is of little physiological significance in the normal individual (Biglieri, 1978).

Cortisol induces sodium retention and potassium excretion, but much less effectively than does aldosterone. Acute treatment with cortisol, unlike that with aldosterone, does not increase net acid secretion (Lemann *et al.*, 1970). In striking contrast to aldosterone, cortisol, under certain circumstances, especially sodium loading, enhances sodium excretion. This may be accounted for by the capacity of cortisol to increase the glomerular filtration rate (GFR). Aldosterone and desoxycorticosterone are ineffective in this regard. Fur-

thermore, cortisol has a significant stimulatory influence on tubular secretory activity.

Impaired water diuresis in response to an administered water load, while not specific for adrenal insufficiency, has been used as a diagnostic criterion. In adrenal insufficiency, GFR is reduced and plasma antidiuretic hormone (ADH) concentration is increased; these factors account for failure to excrete a water load (Ahmed *et al.*, 1967). Administration of cortisol, but not of aldosterone, increases GFR and restores water diuresis (Gill *et al.*, 1962).

Hypercorticism due to administration of large doses of cortisol (or related glucocorticoids) or to excessive secretion of cortisol by the adrenals is sometimes associated with a hypokalemic hypochloremic alkalosis (*see* Chapter 35). However, the changes, particularly the degree of hypokalemia, are moderate in severity and reflect the relatively weak effect of cortisol as compared to aldosterone on electrolyte balance. Muscular weakness associated with glucocorticoid treatment is usually due to a loss of muscle mass rather than of potassium.

Cardiovascular System. The most striking effects of corticosteroids on the cardiovascular system are those that are the consequence of regulation of renal sodium ion excretion. These are seen most vividly in hypocorticism when reduction in blood volume accompanied by increased viscosity can lead to hypotension and cardiovascular collapse. However, the impairment of the cardiovascular system in adrenocortical insufficiency obviously involves additional, poorly understood processes. The corticosteroids exert important actions on the various elements of the circulatory system, including the capillaries, the arterioles, and the myocardium. In the absence of the corticosteroids, there is increased capillary permeability, inadequate vasomotor response of the small vessels, and reduction in cardiac size and output.

An excess of mineralocorticoids occurs in its purest form in *primary aldosteronism*, the result of excessive secretion of this steroid. In this disease the major clinical findings are hypertension and hypokalemia. The hypokalemia is an obvious consequence of the renal effects of aldosterone, but the genesis of the hypertension has not been totally clarified. Development of hypertension requires a prolonged excess of mineralocorticoid and increased sodium intake (Mulrow and Forman, 1972). Hypertension occurs in most cases of Cushing's syndrome but rarely, if at all, as the result of administration of synthetic glucocorticoids lacking mineralocorticoid activity. Steroid-induced hypertension may be the result of prolonged, excessive sodium retention; one hypothesis proposes that this leads to edema within the walls of arterioles, thereby reducing their lumina and increasing peripheral vascular resistance (Tobian, 1960). Another possibility is that salt retention or mineralocorticoids themselves sensitize blood vessels to pressor agents, in particular angiotensin and catecholamines (Brunner *et al.*, 1972; Yard and Kadowitz, 1972). The concentration of renin substrate is elevated in Cushing's syndrome, and this too may play a role (Krakoff *et al.*, 1975). There is also some evidence that ADH plays a role in the pathogenesis of hypertension produced by mineralocorticoids (Share and Crofton, 1982).

Skeletal Muscle. The maintenance of normal function of skeletal muscle requires adequate concentrations of corticosteroids, but excessive amounts of either mineralocorticoids or glucocorticoids lead to abnormalities.

It is well known that one of the outstanding signs of adrenocortical insufficiency is a diminished work capacity of striated muscle. This is manifested in patients with Addison's disease by weakness and fatigue. The most important single factor responsible for this dysfunction appears to be the inadequacy of the circulatory system. Abnormalities in electrolyte balance and carbohydrate metabolism in adrenocortical insufficiency contribute only in small measure to the impairment in skeletal muscle function.

Muscle weakness in primary aldosteronism is in large measure a result of the hypokalemia characteristic of this disease. Glucocorticoids given for prolonged periods in high doses or secreted in abnormal amounts in Cushing's syndrome tend to cause a wasting of skeletal muscle. The mechanism of this is not known. This steroid myopathy is responsible, at least in part, for the weakness and fatigue noted in the syndrome. Steroid-induced myopathy has been reviewed by Mandel (1982).

Central Nervous System. The corticosteroids affect the central nervous system (CNS) in a number of indirect ways; in particular, they maintain normal concentrations of glucose in plasma, an adequate circulation, and the normal balance of electrolytes in the body. The steroids may also have direct effects, but these are as yet poorly defined. An influence of the corticosteroids can be observed on mood, behavior, the EEG, and brain excitability.

Patients with Addison's disease exhibit apathy, depression, and irritability, and some are frankly psychotic. Desoxycorticosterone is ineffective but cortisol is very effective in correcting these abnormalities of psyche and behavior. An array of reactions, varying in degree and kind, is seen in patients to whom glucocorticoids are administered for therapeutic purposes. Most patients respond with elevation in mood, which may be explained in part by the relief of the symptoms of the disease being treated. In some, more definite mood changes occur, characterized by euphoria, insomnia, restlessness, and increased motor activity. A smaller but significant percentage of patients treated with high doses of cortisol become anxious or depressed, and a still smaller percentage exhibit psychotic reactions. There is a high incidence of neuroses and psychoses among patients with Cushing's syndrome. The abnormalities of behavior usually disappear when the corticosteroids are withdrawn or the Cushing's syndrome is effectively treated.

There is usually an increase in the excitability of neural tissue in hypocorticism and a decrease in animals given large doses of desoxycorticosterone; these alterations appear to be related to changes in the concentrations of electrolytes in the brain. In contrast, administration of cortisol increases brain excitability without influencing the concentrations of sodium and potassium in the brain. It is concluded that the influence of desoxycorticosterone on excitability is mediated through its influence on sodium transport, whereas cortisol acts by a different mechanism, presumably mediated by cytoplasmic receptors (McEwen, 1979; Carpenter and Gruen, 1982).

Thresholds for the perception of taste, smell, and sound stimuli are reduced in adrenocortical insufficiency and elevated in hypercorticism. Glucocorticoids restore thresholds to normal, but desoxycorticosterone is without effect (Henkin, 1970).

Formed Elements of Blood. Glucocorticoids tend to increase the hemoglobin and red-cell content of the blood, as evidenced by the frequent occurrence of polycythemia in Cushing's syndrome and a mild, normochromic, normocytic anemia in Addison's disease. The capacity of these steroids to retard erythrophagocytosis may be a factor in the production of polycythemia.

The corticosteroids also affect circulating white cells. Administration of glucocorticoids leads to an increase in the number of polymorphonuclear leukocytes in the blood as the result of an increased rate of entrance into the blood from the marrow and a diminished rate of removal from the circulation (Bishop *et al.*, 1968). In contrast, the lymphocytes, eosinophils, monocytes, and basophils of the blood decrease in number after administration of glucocorticoids. A single dose of cortisol produces a decline of about 70% in circulating lymphocytes and a decline of over 90% in monocytes; this occurs in 4 to 6 hours and lasts for about 24 hours. The decrease in lymphocytes, monocytes, and eosinophils appears to result from redistribution of cells, rather than from their destruction. The cause of the fall in circulating basophils has not been established.

After administration of a glucocorticoid, the thymus-derived lymphocytes (T cells) are decreased proportionately more than those that are derived from the bone marrow (B cells). The profile of cellular responses of the lymphocytes remaining in the blood to various mitogens and antigens is altered when contrasted to that of lymphocytes of untreated subjects. This indicates that subpopulations of lymphocytes are differentially affected by the steroids (*see* Cupps and Fauci, 1982).

Anti-inflammatory Properties. Cortisol and the synthetic analogs of cortisol have the capacity to prevent or suppress the development of the local heat, redness, swelling, and tenderness by which inflammation is recognized. At the microscopic level, they inhibit not only the early phenomena of the inflammatory process (edema, fibrin deposition, capillary dilatation, migration of leukocytes into the inflamed area, and phagocytic activity) but also the later manifestations (capillary proliferation, fibroblast proliferation, deposition of collagen, and, still later, cicatrization).

Although understanding of these effects is unsatisfactory, many observations have been made that have therapeutic relevance and that must be taken into account in explanatory formulations. Perhaps the most important of these for the physician is that corticosteroids inhibit the inflammatory response whether the inciting agent is radiant, mechanical, chemical, infectious, or immunological. In clinical terms, the administration of corticosteroids for their anti-inflammatory effects is palliative therapy; the underlying cause of the disease remains; the inflammatory manifestations are merely suppressed. It is this suppression of inflammation and its consequences that has made the corticosteroids such valuable therapeutic agents—indeed, at times lifesaving. It is also this property that gives them a nearly unique potential for therapeutic disaster. The signs and symptoms of inflammation are expressions of the disease process that are often used by the physician in diagnosis and in evaluating the effectiveness of treatment. These may be missing in patients treated with glucocorticoids. For example, an infection may continue to progress while the patient superficially appears to improve, and a peptic ulcer may perforate without producing clinical signs. This situation has been epitomized in the grimly facetious remark that the corticosteroids, misused, permit a patient to walk all the way to the autopsy room!

Anti-inflammatory effects depend upon the direct local action of the steroids. The most important factor in the anti-inflammatory action of gluco-

corticoids may be their ability to inhibit the recruitment of neutrophils and monocyte-macrophages into the affected area (Parrillo and Fauci, 1979). Treatment with glucocorticoids decreases the adherence of neutrophils to nylon fibers, and this may reflect a diminished tendency of these cells to adhere to capillary endothelial cells in areas of inflammation (MacGregor, 1977).

In the inflammatory response of delayed sensitivity reactions, lymphocytes previously sensitized to a particular antigen encounter the antigen within a tissue at a site destined to be the location of the inflammatory response. These lymphocytes, activated by the antigen, begin production of a number of soluble factors, *lymphokines*, that control the cellular response. Among the lymphokines is the macrophage migration inhibitory factor (MIF), which causes an accumulation of nonsensitized macrophages in the area by inhibiting their mobility (Bloom and Bennett, 1966). Glucocorticoids do not affect the production of MIF by lymphocytes that have been activated by an appropriate antigen, but the steroids do block the effect of MIF on macrophages; that is, the movement of these cells is no longer impeded, and they do not accumulate locally (Balow and Rosenthal, 1973).

Low concentrations of glucocorticoids inhibit the formation of plasminogen activator by neutrophils (Granelli-Piperano *et al.*, 1977). This enzyme converts plasminogen to plasmin (fibrinolysin), which is thought to facilitate the entrance of leukocytes into areas of inflammation by hydrolysis of fibrin and other proteins. There is also substantial evidence that the glucocorticoids induce the synthesis of a protein that inhibits phospholipase A_2 and thereby diminishes release of arachidonic acid from phospholipids. This decreases formation of prostaglandins, leukotrienes, and related compounds, such as prostaglandin endoperoxides and thromboxane, which may play an important role in chemotaxis and inflammation (Blackwell *et al.*, 1980; Hirata *et al.*, 1980; *see* Chapter 28). As a result of studies on a particular strain of mouse fibroblasts maintained in culture, it was thought that glucocorticoids inhibit the growth of such cells. It is now evident that human fibroblasts are resistant to glucocorticoids; the suppression of late stages of inflammation by inhibition of fibroblast proliferation is probably not a valid model for the anti-inflammatory effects of glucocorticoids (Priestley and Brown, 1980).

Lymphoid Tissue and Immune Responses. Addison was the first to observe the increase in mass of lymphoid tissue that accompanies adrenocortical insufficiency; there is also a lymphocytosis. In contrast, Cushing's syndrome is characterized by lymphocytopenia and decreased mass of lymphoid tissue.

While glucocorticoids cause a rapid lysis of lymphatic tissue in rats and mice, there is no evidence of a comparable effect in man (Claman, 1972). This implies that the changes in lymphoid tissue seen in man in chronic hypercorticosteroid or hypocorticosteroid states must result from changes in rates of cellular formation or destruction that become manifest over a prolonged period of time. As noted above, the acute effects of steroids on circulating lymphocytes are due to sequestration from the blood rather than to lymphocytolysis. Although glucocorticoids do not produce a sudden massive lysis of lymphoid tissue in man, cells of acute lymphoblastic leukemia and, in some cases, cells of other lymphatic malignancies are destroyed by glucocorticoids in a manner presumed to be analogous to that which occurs in lymphoid tissue of rodents.

As noted, glucocorticoids and ACTH modify the clinical course of a variety of diseases in which hypersensitivity is believed to play an important role. Although massive doses of methylprednisolone have been shown to cause a modest fall in the concentration of IgG in the plasma of human volunteers, these same subjects produced antibody normally in response to antigenic stimuli (Butler, 1975). On the whole, there is no convincing evidence that the therapeutic use of the corticosteroids has a significant effect on the titer of circulating antibodies, either IgG or IgE, that play a major role in allergic and autoimmune states. Metabolism of complement is likewise probably not significantly affected (Claman, 1975). This is true in spite of the fact that the symptoms of the diseases are often alleviated dramatically by the steroids. It is also now believed that in clinical situations in which the glucocorticoids are used to prevent the consequences of cell-mediated (delayed hypersensitivity) immune reactions, for example, graft rejection, the steroids do not interfere with the development of immune lymphatic cells that are capable of eliciting an inflammatory response upon contact with the sensitizing antigen. Rather, they suppress the inflammatory response, apparently by inhibiting recruitment of leukocytes into the region of contact with the foreign antigen (*see* above; Weston *et al.*, 1973).

Circulating monocytes from individuals who are receiving glucocorticoids display an impaired ability to kill microorganisms, although the process of phagocytosis is not defective (Rinehart *et al.*, 1975).

There are numerous reports of effects of glucocorticoids on lymphatic cells *in vitro*. Unfortunately, many of these effects are observed at unrealistically high concentrations of steroids, and their significance is unclear. One example of a response that is inhibited by appropriately low concentrations of glucocorticoids *in vitro* is the proliferation of T cells stimulated by mitogens or mixed leukocyte cultures. This effect is the result of inhibition of the release of interleukin 1 by macrophages. Deficiency of interleukin 1 precludes formation of interleukin 2, the immediate stimulus for proliferation of T cells (Gillis *et al.*, 1979; Smith, 1980).

Growth and Cell Division. Pharmacological doses of glucocorticoids retard or interrupt the growth of children, indicating an adverse effect on the epiphyseal cartilage. Inhibition of growth is a rather widespread effect of the glucocorticoids in tissues of laboratory animals. For example, they inhibit cell division or the synthesis of DNA in thymocytes; normal, developing, and regenerating liver; gastric mucosa; developing brain; developing lung; and human epidermis. Nevertheless, this effect is somewhat selective, and corticosteroids do not characteristically produce the bone-marrow depression or the enteritis that follows exposure to nonspecific antimitotic agents. The mechanism of this effect of the steroids is not known.

ABSORPTION, TRANSPORT, METABOLISM, AND EXCRETION

Absorption. Cortisol and numerous congeners, including synthetic analogs, are effective when given by mouth. Desoxycorticosterone acetate is unusual in that it is ineffective by this route.

Water-soluble esters of cortisol and its synthetic congeners are administered intravenously in order to achieve high concentrations in body fluids rapidly. More prolonged effects are obtained by intramuscular injection of suspensions of cortisol, congeners, and esters. Minor changes in chemical structure may result in large changes in the rate of absorption, time of onset of effect, and duration of action.

Glucocorticoids are absorbed from sites of local application such as synovial spaces, the conjunctival sac, and the skin. The absorption may be sufficient, when administration is chronic or large areas of skin are involved, to cause systemic effects, including adrenocortical suppression.

Transport, Metabolism, and Excretion. In the plasma, 90% or more of the cortisol is reversibly bound to protein under normal circumstances. The binding is accounted for by two proteins. One, corticosteroid-binding globulin, is a glycoprotein; the other is albumin. The globulin has high affinity but low total binding capacity, while albumin has low affinity but relatively large binding capacity. Consequently, at low or normal concentrations of corticosteroids most of the hormone is bound to globulin. When the amount of corticosteroid is increased, concentrations of both free and albumin-bound steroid increase with little change in the concentration of that bound to the globulin. Corticosteroids compete with each other for binding sites on the corticosteroid-binding globulin. Cortisol has high affinity; glucuronide-conjugated steroid metabolites and aldosterone have low affinities.

During pregnancy and during estrogen treatment in both sexes, corticosteroid-binding globulin, total plasma cortisol, and free cortisol increase several-fold. The physiological significance of these facts is not known. The free hormone as opposed to the protein-bound steroid is biologically active, available for hepatic metabolism, and may be excreted by the kidney.

All the biologically active adrenocortical steroids and their synthetic congeners have a double bond in the 4,5 position and a ketone group at C 3. Reduction of the 4,5 double bond can occur at both hepatic and extrahepatic sites and yields an inactive substance. Subsequent reduction of the 3-ketone substituent to a 3-hydroxyl to form tetrahydrocortisol has been demonstrated only in liver. Most of the ring-A–reduced metabolites are enzymatically coupled through the 3-hydroxyl with sulfate or with glucuronic acid to form water-soluble sulfate esters or glucuronides, and they are excreted as such. These conjugation reactions occur principally in liver and to some extent in kidney.

Reversible oxidation of the 11-hydroxyl group has been demonstrated to occur slowly in a variety of extrahepatic tissues and rapidly in liver. Corticosteroids with an 11-ketone substituent require reduction to 11-hydroxyl compounds for their biological

activity. Reduction of the 20-ketone group to a 20-hydroxyl configuration yields a substance having little, if any, biological activity. Corticosteroids with a hydroxyl group at C 17 undergo an oxidation that yields 17-ketosteroids and a two-carbon fragment. These 17-ketosteroids are totally lacking in corticosteroid activity but, in a few instances, have weak androgenic properties.

When radioactive-carbon, ring-labeled steroids are injected intravenously in man, most of the radioisotope is recovered in the urine within 72 hours. Neither biliary nor fecal excretion is of any quantitative importance in man. It has been estimated that the liver metabolizes at least 70% of the cortisol secreted.

The metabolism of cortisol has been studied more extensively than that of all other corticosteroids, and it is generally assumed that the metabolism of its congeners and synthetic derivatives is qualitatively similar. Cortisol has a plasma half-life of about 1.5 hours. The metabolism of corticosteroids is greatly slowed by introduction of the 1,2 double bond or a fluorine atom into the molecule, and the half-life is correspondingly prolonged.

Clinical laboratories measure urinary cortisol and metabolites with reduced ring A as "17-hydroxycorticosteroids." These compounds and those where the ketone at carbon 20 has been reduced are included in the group referred to as "17-ketogenic steroids." The urinary metabolites that have lost their side chain contribute to the "17-ketosteroids."

STRUCTURE-ACTIVITY RELATIONSHIP

Cortisone was the first corticosteroid used for its anti-inflammatory effect. Modifications of structure have led to increases in the ratio of anti-inflammatory to sodium-retaining potency, such that in a number of presently available compounds electrolyte effects are of no serious consequence, even at the highest doses used. However, in all compounds studied to date, effects on inflammation and on carbohydrate and protein metabolism have paralleled one another. It seems very likely that effects on inflammation and metabolism are mediated by the same type of receptor.

Changes in molecular structure may bring about changes in biological potency as a result of alterations in absorption, protein binding, rate of metabolic transformation, rate of excretion, ability to traverse membranes, and intrinsic effectiveness of the molecule at its site of action. In the following paragraphs, modifications of the pregnane nucleus that have been of value in therapeutic agents are described. The molecular sites of alteration are shown in Figure 63–4 in bold lines and letters. Table 63–3 lists the effects of the modifications discussed relative to cortisol. As indicated above, relative potencies vary to some extent with different conditions of bioassay.

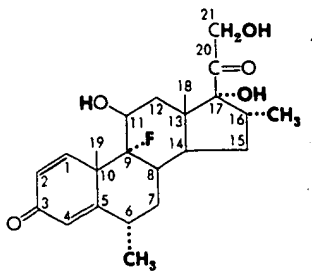


Figure 63–4. *Structure-activity relationship of adrenocorticosteroids.*

Light lines and letters indicate structural features common to compounds having anti-inflammatory action. Bold lines and letters indicate modifications that enhance or suppress characteristic activities. (After Liddle, 1961. Courtesy of *Clinical Pharmacology and Therapeutics*.)

Ring A. The 4,5 double bond and the 3-ketone are both necessary for typical adrenocorticosteroid activity. Introduction of a 1,2 double bond, as in prednisone or prednisolone, enhances the ratio of carbohydrate-regulating potency to sodium-retaining potency by selectively increasing the former. In addition, prednisolone is metabolized more slowly than cortisol.

Ring B. 6α-Substitution has unpredictable effects. In the particular instance of cortisol, 6α-methylation increases anti-inflammatory, nitrogen-wasting, and sodium-retaining effects in man. In contrast, 6α-methylprednisolone has slightly greater anti-inflammatory potency and less electrolyte-regulating potency than prednisolone. Fluorination in the 9α position enhances all biological activities of the corticosteroids, apparently by its electron-withdrawing effect on the 11β-hydroxy group.

Ring C. The presence of an oxygen function at C 11 is indispensable for significant anti-inflammatory and carbohydrate-regulating potency (cortisol versus 11-desoxycortisol) but is not necessary for high sodium-retaining potency, as demonstrated by desoxycorticosterone.

Ring D. 16-Methylation or hydroxylation eliminates the sodium-retaining effect but only slightly modifies potency with respect to effects on metabolism and inflammation.

All presently used anti-inflammatory steroids are 17α-hydroxy compounds. Although some carbohydrate-regulating and anti-inflammatory effects may occur in 17-desoxy compounds (cortisol versus corticosterone), the fullest expression of these activities requires the presence of the 17α-hydroxy substituent.

All natural corticosteroids and most of the active synthetic analogs have a 21-hydroxy group. While some glycogenic and anti-inflammatory activities may occur in its absence, its presence is required for significant sodium-retaining activity.

Table 63–3. RELATIVE POTENCIES AND EQUIVALENT DOSES OF CORTICOSTEROIDS

COMPOUND	RELATIVE ANTI-INFLAMMATORY POTENCY	RELATIVE SODIUM-RETAINING POTENCY	DURATION OF ACTION *	APPROXIMATE EQUIVALENT DOSE † (*mg*)
Cortisol (Hydrocortisone)	1	1	S	20
Tetrahydrocortisol	0	0	—	—
Prednisone (Δ^1-Cortisone)	4	0.8	I	5
Prednisolone (Δ^1-Cortisol)	4	0.8	I	5
6α-Methylprednisolone	5	0.5	I	4
Fludrocortisone (9α-Fluorocortisol)	10	125	S	—
11-Desoxycortisol	0	0	—	—
Cortisone (11-Dehydrocortisol)	0.8	0.8	S	25
Corticosterone	0.35	15	S	—
Triamcinolone (9α-Fluoro-16α-hydroxyprednisolone)	5	0	I	4
Paramethasone (6α-Fluoro-16α-methylprednisolone)	10	0	L	2
Betamethasone (9α-Fluoro-16β-methylprednisolone)	25	0	L	0.75
Dexamethasone (9α-Fluoro-16α-methylprednisolone)	25	0	L	0.75

* S = Short or 8- to 12-hour biological half-life; I = intermediate or 12- to 36-hour biological half-life; L = long or 36- to 72-hour biological half-life (*see* Rose and Saccar, 1978).

† These dose relationships apply only to oral or intravenous administration; relative potencies may differ greatly when injected intramuscularly or into joint spaces.

PREPARATIONS AND ROUTES OF ADMINISTRATION

Organic chemists have synthesized a bewildering number of modified adrenocorticosteroids, many of which share the same properties and differ only with respect to absolute dosage. At the outset it should be reemphasized that, whereas a clear separation has been made between mineralocorticoids and glucocorticoids, there is no member of the latter group that is unique with respect to a separation of therapeutic and toxic effects. A working knowledge of a small number of preparations is sufficient for nearly every clinical purpose.

Corticosteroids are administered orally, parenterally (intravenous, intramuscular, subcutaneous, intrasynovial, and intralesional routes), and topically (dermal ointments, creams, and lotions; ophthalmic ointments and solutions; respiratory aerosols; enemas). Some absorption into the systemic circulation occurs with all forms of topical administration. In the case of most aerosols, absorption is virtually equivalent to that from parenteral or oral administration. Adrenocortical suppression can occur with applications of steroids to the conjunctival sac and to the skin. Absorption from the skin is especially marked when the steroid is applied under plastic film over a large surface area.

Information on available steroid preparations is presented in Table 63–4.

TOXICITY OF ADRENOCORTICAL STEROIDS

Two categories of toxic effects are observed in the therapeutic use of adrenocorticosteroids: those resulting from *withdrawal* and those resulting from *continued use of large doses*. Acute adrenal insufficiency results from too rapid withdrawal of corticosteroids after prolonged therapy. Protocols for discontinuing corticosteroid therapy in patients who have been subjected to suppressive therapy for long periods have been described by Harter and associates (1963) and Byyny (1976). There is a characteristic corticosteroid withdrawal syndrome, consisting in fever, myalgia, arthralgia, and malaise, which may be extremely difficult to distinguish from "reactivation" of rheumatoid arthritis or rheumatic fever (Amatruda *et al.*, 1960). Pseudotumor cerebri with papilledema is a rare reaction that follows reduction or withdrawal of corticosteroid therapy (Levine and Leopold, 1973).

Table 63–4. PREPARATIONS OF ADRENOCORTICAL STEROIDS AND THEIR SYNTHETIC ANALOGS *

NONPROPRIETARY NAME AND TRADE NAMES	ORAL FORMS	INJECTABLE FORMS	OTHERS [1]
Desoxycorticosterone acetate [2] (DOCA ACETATE, PERCORTEN ACETATE)	—	5 mg/ml (oil) 125 mg (pellets)	—
Desoxycorticosterone pivalate (PERCORTEN PIVALATE)	—	25 mg/ml (susp.)	—
Fludrocortisone acetate [3] (FLORINEF ACETATE)	0.1 mg	—	—
Cortisol [2] (hydrocortisone) (CORTEF, HYDROCORTONE)	5–20 mg	25, 50 mg/ml (susp.)	TA: 0.125–2.5% 100-mg/60-ml enema
Cortisol (hydrocortisone) acetate (CORTEF ACETATE, HYDROCORTONE ACETATE, others)	—	25, 50 mg/ml (susp.)	TA: 0.5–2.5% 15-, 25-mg suppositories 10% rectal foam
Cortisol (hydrocortisone) cypionate (CORTEF FLUID)	2 mg/ml (susp.)	—	—
Cortisol (hydrocortisone) sodium phosphate (HYDROCORTONE PHOSPHATE)	—	50 mg/ml	—
Cortisol (hydrocortisone) sodium succinate (A-HYDROCORT, SOLU-CORTEF)	—	100–1000 mg (powder)	—
Beclomethasone dipropionate [4] (BECLOVENT, VANCERIL)	—	—	I: 42 μg per dose
Betamethasone [3] (CELESTONE)	0.6 mg 0.6 mg/5 ml (syrup)	—	—
Betamethasone benzoate (BENISONE, UTICORT)	—	—	TA: 0.025%
Betamethasone dipropionate (DIPROSONE)	—	—	TA: 0.05, 0.1%
Betamethasone sodium phosphate and acetate (CELESTONE SOLUSPAN)	—	6 mg/ml (susp.)	—
Betamethasone valerate (BETA-VAL, VALISONE)	—	—	TA: 0.01, 0.1%
Cortisone acetate [3] (CORTONE ACETATE)	5–25 mg	25, 50 mg/ml (susp.)	—
Dexamethasone [3] (DECADRON, others)	0.25–6.0 mg 0.5 mg/5 ml (elixir) 0.5 mg/0.5 ml (soln.)	—	TA: 0.01, 0.1% O: 0.1%
Dexamethasone acetate (DECADRON-LA, others)	—	2–16 mg/ml (susp.)	—
Dexamethasone sodium phosphate (DECADRON PHOSPHATE, HEXADROL PHOSPHATE, others)	—	4–24 mg/ml	TA: 0.1% O: 0.05, 0.1% I: 100 μg per dose
Methylprednisolone [3] (MEDROL)	2–32 mg	—	—

Table 63–4. PREPARATIONS OF ADRENOCORTICAL STEROIDS AND THEIR SYNTHETIC ANALOGS * (Continued)

NONPROPRIETARY NAME AND TRADE NAMES	ORAL FORMS	INJECTABLE FORMS	OTHERS [1]
Methylprednisolone acetate (DEPO-MEDROL, MEDROL ACETATE, others)	—	20–80 mg/ml (susp.)	TA: 0.25, 1% 40-mg/unit enema
Methylprednisolone sodium succinate (A-METHAPRED, SOLU-MEDROL)	—	40–1000 mg (powder)	—
Paramethasone acetate [3] (HALDRONE)	1, 2 mg	—	—
Prednisolone [3] (DELTA-CORTEF, others)	1, 5 mg	—	—
Prednisolone acetate (ECONOPRED, others)	—	25–100 mg/ml (susp.)	O: 0.12–1%
Prednisolone sodium phosphate (HYDELTRASOL, others)	—	20 mg/ml	O: 0.125–1%
Prednisolone tebutate (HYDELTRA-T.B.A.)	—	20 mg/ml (susp.)	—
Prednisone [3] (DELTASONE, others)	1–50 mg 1 mg/ml (syrup)	—	—
Triamcinolone [3] (ARISTOCORT, KENACORT)	1–16 mg	—	—
Triamcinolone acetonide (KENALOG, others)	—	10, 40 mg/ml (susp.)	TA: 0.025–0.5% I: 100 μg per dose
Triamcinolone diacetate (ARISTOCORT, KENACORT DIACETATE, others)	2, 4 mg/5 ml (syrup)	25, 40 mg/ml (susp.)	—
Triamcinolone hexacetonide (ARISTOSPAN)	—	5, 20 mg/ml (susp.)	—
Amcinonide [4] (CYCLOCORT)	—	—	TA: 0.1%
Clocortolone pivalate [4] (CLODERM)	—	—	TA: 0.1%
Desonide [4] (TRIDESILON)	—	—	TA: 0.05%
Desoximetasone [4] (TOPICORT)	—	—	TA: 0.05, 0.25%
Diflorasone diacetate [4] (FLORONE, MAXIFLOR)	—	—	TA: 0.05%
Flumethasone pivalate [4] (LOCORTEN)	—	—	TA: 0.03%
Fluocinolone acetonide [4] (FLUONID, SYNALAR, others)	—	—	TA: 0.01–0.2%
Fluocinonide [4] (LIDEX)	—	—	TA: 0.05%
Fluorometholone [4] (FML LIQUIFILM)	—	—	O: 0.1%

Table 63–4. PREPARATIONS OF ADRENOCORTICAL STEROIDS AND THEIR SYNTHETIC ANALOGS * (Continued)

NONPROPRIETARY NAME AND TRADE NAMES	ORAL FORMS	INJECTABLE FORMS	OTHERS [1]
Flurandrenolide [4] (CORDRAN)	—	—	TA: 0.025, 0.05%; 4 μg/sq cm tape
Halcinonide [4] (HALOG)	—	—	TA: 0.025, 0.1%
Medrysone [4] (HMS LIQUIFILM)	—	—	O: 1%

* Preparations above the double line are intended for use as mineralocorticoids.

[1] TA = topical application to skin or mucous membranes in creams, solutions, ointments, gels, lotions, or aerosols. O = ophthalmic solution, suspension, or ointment. I = nasal or oral inhalation.

[2] *See* Figure 63—3 for structure.

[3] *See* Table 63—3 for chemical name.

[4] Beclomethasone, 9α-chloro,16β-methylprednisolone,17,21-dipropionate; amcinonide, 9α-fluoro,16α-hydroxyprednisolone, cyclic 16,17-acetal with cyclic pentanone,21-acetate; clocortolone, $\Delta^{1,2}$,6α-fluoro,9α-chloro,16α-methylcorticosterone 21-pivalate; desonide, 16α-hydroxyprednisolone, cyclic 16,17-acetal with acetone; desoximetasone, $\Delta^{1,2}$,9α-fluoro,16α-methylcorticosterone; diflorasone diacetate, 6α,9α-difluoro,16β-methylprednisolone, 17,21-diacetate; flumethasone, 6α,9α-difluoro,16α-methylprednisolone; fluocinolone, 6α,9α-difluoro, 16α-hydroxyprednisolone,16,17-acetal with acetone; fluocinonide, 6α,9α-difluoro,16α-hydroxyprednisolone,16,17-acetal with acetone,21-acetate; fluorometholone, $\Delta^{1,2}$,9α-fluoro,6α-methyl, 11β,17-dihydroxyprogesterone; flurandrenolide, 6α-fluoro,16α-hydroxycortisol,16,17-acetal with acetone; halcinonide, 21-chloro,9α-fluoro,11β,16α,17-trihydroxypregn-4-ene-3,20-dione,16,17-acetal with acetone; medrysone, 11β-hydroxy,6α-methylprogesterone.

The use of corticosteroids for days or a few weeks does not lead to adrenal insufficiency upon cessation of treatment, but prolonged therapy with corticosteroids may result in suppression of pituitary-adrenal function that can be slow in returning to normal. Graber and coworkers (1965) found that the processes of recovery of normal pituitary and adrenal function required 9 months in some patients. During this recovery period and for an additional 1 to 2 years, the patient may need to be protected during stressful situations, such as surgery or severe infections, by the administration of corticosteroids. Dixon and Christy (1980) have discussed the complex clinical problems that can be provoked by withdrawal from steroid therapy.

In addition to pituitary-adrenal suppression, the principal complications resulting from prolonged therapy with corticosteroids are fluid and electrolyte disturbances; hyperglycemia and glycosuria; increased susceptibility to infections, including tuberculosis; peptic ulcers, which may bleed or perforate; osteoporosis; a characteristic myopathy; behavioral disturbances; posterior subcapsular cataracts; arrest of growth; and Cushing's habitus, consisting in "moon face," "buffalo hump," enlargement of supraclavicular fat pads, "central obesity," striae, ecchymoses, acne, and hirsutism.

Hypokalemic alkalosis and *edema* are infrequently encountered in patients who are treated with synthetic corticosteroid congeners and almost never in patients taking the 16-substituted compounds. *Glycosuria* can usually be managed with diet and/or insulin, and its occurrence should not be an important factor in the decision to continue corticosteroid therapy or to initiate it in diabetic patients.

Increased susceptibility to infection in patients treated with corticosteroids is not specific for any particular bacterial or fungal pathogen. If infection develops in a patient treated with corticosteroids, the dose may be maintained or increased and the best available treatment for the infection vigorously administered. Corticosteroid therapy may be initiated in patients having known infections of some consequence if effective, specific chemotherapy can be administered concomitantly with the hormones. However, in these circumstances the physician should be confident that the corticosteroid is needed, that the pathogen has been identified, and that chemotherapy will be effective.

Peptic ulceration is an occasional complication of corticosteroid therapy. The high incidence of hemorrhage and perforation in these ulcers and the insidious nature of their development make them severe therapeutic problems. However, there has

been disagreement about the incidence of these ulcers, and some studies have concluded that evidence does not support an association between peptic ulcers and treatment with glucocorticoids (Conn and Blitzer, 1976). It is also not known whether there is an interaction between glucocorticoids and nonsteroidal anti-inflammatory drugs, such as aspirin, which, by themselves, can cause ulcers. In a recent survey of the literature, Messer and associates (1983) concluded that steroid therapy approximately doubles the risk of ulcer (*see also* Spiro, 1983).

Myopathy, characterized by weakness of the proximal musculature of arms and legs and of their associated shoulder and pelvic muscles, is occasionally seen in patients taking large doses of corticosteroids. It may occur soon after treatment is begun and be sufficiently severe to prevent ambulation. It is not specific for synthetic corticosteroid congeners, for it is found in endogenous Cushing's syndrome. It is a serious complication and an indication for withdrawal of therapy. Recovery may be slow and incomplete (*see* Mandel, 1982).

Behavioral disturbances may take various forms, for example, nervousness, insomnia, changes in mood or psyche, and psychopathies of the manic-depressive or schizophrenic type. Suicidal tendencies are not uncommon. It is no longer believed that previous psychiatric problems predispose to behavioral disturbances during therapy with glucocorticoids. Conversely, the absence of a history of psychiatric illness is no guarantee against the occurrence of psychosis during hormonal therapy.

Posterior subcapsular cataracts have been reported in children receiving corticosteroid therapy. Many patients with rheumatoid arthritis who receive 20 mg of prednisone per day for 4 years develop cataracts (Levine and Leopold, 1973); it is possible that patients with this disease are particularly susceptible to this complication. The problem of corticosteroid-induced cataracts has been reviewed by Lubkin (1977).

Osteoporosis and *vertebral compression fractures* are frequent serious complications of corticosteroid therapy in patients of all ages. Ribs and vertebrae, bones with a high degree of trabecular structure, are generally the most severely affected. Glucocorticoids appear to inhibit the activities of osteoblasts directly, and, because of their inhibition of calcium absorption by the intestine, glucocorticoids cause an increased secretion of parathyroid hormone (PTH). PTH stimulates the activity of osteoclasts (Hahn, 1978); thus, there is both decreased formation and increased resorption of bone. As noted above, corticosteroids also increase calcium excretion by the kidney. Osteoporosis is an indication for withdrawal of therapy and should be looked for regularly in radiographs of the spine in patients taking glucocorticoids for longer than a few months. Unfortunately, significant loss of bone must occur before it is apparent from radiography. The possibility of development of osteoporosis should be an important consideration in initiating and managing corticosteroid therapy, especially in postmenopausal women (*see* Baylink, 1983).

Inhibition or arrest of growth can result from the administration of relatively small doses of glucocorticoids to children. This cannot be overcome with exogenous human growth hormone (Morris *et al.*, 1968). The widespread inhibitory effect of the glucocorticoids on DNA synthesis and cell division discussed above is apparently responsible. Inhibition of growth by glucocorticoids has been reviewed by Loeb (1976).

THERAPEUTIC USES

With the exception of substitution therapy in deficiency states, the use of corticosteroids and their congeners in disease is largely empirical. From the experience accumulated since the introduction of glucocorticoids for clinical use, at least six therapeutic principles may be abstracted, as follows: (1) for any disease, in any patient, the appropriate dose to achieve a given therapeutic effect must be determined by trial and error and must be reevaluated from time to time as the stage and the activity of the disease alter; (2) a *single* dose of corticosteroid, even a large one, is virtually without harmful effects; (3) a few days of corticosteroid therapy, in the absence of specific contraindications, is unlikely to produce harmful results except at the most extreme dosages; (4) as corticosteroid ther-

apy is prolonged over periods of weeks or months, and to the extent that the dose exceeds the equivalent of substitution therapy, the incidence of disabling and potentially lethal effects increases; (5) except in adrenal insufficiency, the administration of corticosteroids is neither etiological nor curative therapy but only palliative by virtue of the anti-inflammatory effects; and (6) abrupt cessation of prolonged, high-dosage corticosteroid therapy is associated with a significant risk of adrenal insufficiency of sufficient severity to be threatening to life.

Translated into the terms of clinical practice, these general principles are equivalent to the following rules. When corticosteroids are to be administered over long periods, the dose must be the smallest one that will achieve a desired effect. This dose must be found by trial and error. Where the goal of therapy is relief of painful or distressing symptoms not associated with an immediately life-threatening disease, for example, rheumatoid arthritis, the initial dose should be small and gradually increased until pain or distress has been reduced to tolerable levels. Complete relief is not sought. At frequent intervals the dose should be gradually reduced until the development of more severe symptoms signals that the minimal acceptable dose has been found. When therapy is directed at an immediately life-threatening state, for example, pemphigus, the initial dose should be a large one, estimated to achieve, almost with certainty, control of the crisis. If some benefit is not observed in a short time, the dose should be doubled or tripled. When potentially lethal disease is controlled by large amounts of corticosteroid, reduction of the dose should be carried out under conditions that permit frequent, accurate observations of the patient. Under these circumstances it is essential to assess constantly the relative dangers of therapy and of the disease being treated.

The apparently innocuous character of a single administration of corticosteroid in amounts within the conventional therapeutic range justifies its use without a definite diagnosis for crises in which there exists some probability that life is threatened by primary adrenal or pituitary insufficiency. If one of these conditions is present, a single intravenous injection of a soluble corticosteroid may prevent immediate death and allow time for diagnostic procedures.

Short courses of systemic corticosteroids in large doses may properly be given for diseases that do not threaten life, in the absence of specific contraindications. The general rule is that long courses of therapy at high dosage should be reserved for life-threatening disease. On occasion, and for definite cause, when the patient is threatened with permanent disability, this rule is justifiably violated.

It is not possible to define the precise dose of glucocorticoids that will produce pituitary and adrenocortical suppression in a given patient, since there is considerable variation. In general, the higher the dose and the more prolonged the therapy, the greater is the likelihood of suppression.

Harter and associates (1963) suggested that some dissociation of therapeutic effects from certain undesirable metabolic effects can be achieved by the administration of a single large dose of corticosteroids every other day, in contrast to the usual daily multiple-dose schedule. A single dose every other day or at even longer intervals is acceptable therapy for some, but not all, patients with a variety of diseases modified by corticosteroid therapy. When this therapeutic regimen is possible, the degree of suppression of the pituitary and adrenal cortex can be minimized. Steroids that are long acting are not suitable for use by this dosage schedule.

Substitution Therapy. Insufficiency of secretion of the adrenal cortex results from structural or functional lesions of the adrenal cortex itself or from structural or functional lesions of the anterior pituitary. In either case, the patient may present with acute, catastrophic adrenal insufficiency (adrenal crisis) or chronic adrenal insufficiency. When the adrenal itself is the site of the lesion, all elements of normal adrenal secretion may be reduced or absent or the deficiency may be selective for one or more components of secretion.

Acute Adrenal Insufficiency. This disease is characterized by gastrointestinal symptoms, dehydration, weakness, lethargy, and hypotension. It is usually associated with disorders of the adrenal rather than the pituitary, although exceptions occur. It frequently follows abrupt withdrawal of high doses of corticosteroids.

The immediate needs of such patients are water, sodium, chloride, glucose, cortisol, and appropriate therapy for precipitating causes, for example,

infection, trauma, or hemorrhage. Inasmuch as these patients have a diminished capacity for a water diuresis and have often undergone some degree of cellular hydration, they are susceptible to water intoxication. The principal intravenous fluid should be isotonic sodium chloride solution. Glucose is required for nutrition and to prevent or treat hypoglycemia, but it should be given intravenously in isotonic sodium chloride solution. The total amount of intravenous fluid administered during the first 24 hours should not, in most instances, exceed 5% of ideal body weight. The patient should be monitored for evidence of rising venous pressure and pulmonary edema, because the functional capacity of the cardiovascular system is reduced by adrenocortical insufficiency. Cortisol (hydrocortisone) sodium succinate or cortisol sodium phosphate must be given in the intravenous fluids at a rate of 100 mg every 8 hours, following an initial intravenous injection of 100 mg. This provides a quantity of cortisol that is equal to the maximal daily rate of secretion in response to stress. In the period of transition from intravenous fluid therapy to normal diet and activity, intramuscular cortisol sodium succinate or sodium phosphate may be used in a dose of 25 mg every 6 or 8 hours.

For the treatment of suspected but unconfirmed acute adrenal insufficiency, 4 mg of dexamethasone sodium phosphate should be substituted for cortisol. In addition, ACTH (5 units per hour) should be given. Concentrations of cortisol and aldosterone in plasma and 17-hydroxycorticosteroids in urine are determined at the outset and at intervals during the course of treatment. A failure to obtain a response to ACTH (stimulation of steroid secretion) is diagnostic of adrenal insufficiency. A lack of response in terms of aldosterone indicates failure of the zona glomerulosa.

Chronic Adrenal Insufficiency. This disease results from adrenal surgery or destructive lesions of the adrenal cortex. It requires the administration of cortisol, 20 to 30 mg per day in divided doses. A common dose schedule is 20 mg on arising and 10 mg in the late afternoon. The circadian pattern of ACTH concentrations should be measured. If it rises above normal, multiple doses of cortisol may be required. Most patients will also require a potent mineralocorticoid. The most convenient drug to use for this purpose is fludrocortisone acetate. The usual adult dose is 0.1 to 0.2 mg daily. Some patients do not need a mineralocorticoid and are adequately treated with cortisone and generous dietary salt. Therapy is guided by the patient's sense of well-being, alertness, appetite, weight, muscular strength, pigmentation, blood pressure, and freedom from orthostatic hypotension.

Congenital Adrenal Hyperplasia. This is a familial disorder in which activity of one of several enzymes required for biosynthesis of corticosteroids is deficient. With diminished or absent production of cortisol, aldosterone, or both, and consequent lack of inhibitory feedback, the adrenal cortex is stimulated to the overproduction of other hormonally active steroids. The clinical presentation, laboratory findings, and treatment depend on which of the six enzyme deficiencies thus far described is responsible. Only the syndrome of 21-hydroxylase deficiency will be described here.

In about 90% of the patients with congenital adrenal hyperplasia there is a deficiency of 21-hydroxylase activity. When the deficiency is only partial, the usual case, cortisol is secreted at normal rates as a result of continuous hypersecretion of ACTH, with consequent overproduction of adrenal androgens and their precursors. Aldosterone secretion is approximately normal. Female children undergo virilization, female "pseudohermaphroditism," and male children show precocious development of secondary sex characteristics, "macrogenitosomia." Linear growth is accelerated in childhood, but the height at maturity is reduced by premature closure of the epiphyses.

In about 30% of patients with 21-hydroxylase deficiency, the enzymatic defect is sufficiently severe to compromise increased aldosterone secretion in response to a hypovolemic stimulus. Such patients are unable to conserve sodium normally, in addition to manifesting androgenic effects (Bongiovanni *et al.*, 1967).

All patients with congenital adrenal hyperplasia resulting from a 21-hydroxylase deficiency require substitution therapy with cortisol or a suitable congener, and those with a salt-losing tendency require, in addition, a sodium-retaining steroid. The usual oral dose of cortisol is about 0.6 mg/kg daily in four divided doses, the last one being given as late as possible in order to maintain pituitary suppression overnight. When parenteral substitution therapy is necessary, cortisone acetate may be given intramuscularly every other day. The mineralocorticoid usually given is fludrocortisone acetate, 0.05 to 0.2 mg per day. Therapy is guided by gain in weight and height, by excretion of urinary 17-ketosteroids, and by blood pressure. Sudden spurts of linear growth may indicate inadequate pituitary suppression and excessive androgen secretion, whereas growth failure suggests overtreatment.

A number of rare forms of congenital adrenal hyperplasia are known in which enzyme deficiencies of the adrenal cortex, with similar defects of the gonads, result in clinical and laboratory findings very different from those described above for 21-hydroxylase deficiency. The types described thus far are: "desmolase" deficiency (Camacho *et al.*, 1968), 3β-hydroxysteroid dehydrogenase deficiency (Bongiovanni *et al.*, 1967), 17α-hydroxylase deficiency (Goldsmith *et al.*, 1968), 11β-hydroxylase deficiency (Bongiovanni *et al.*, 1967), and 18-hydroxylase deficiency (David *et al.*, 1968). The clinical and laboratory findings and the treatment in these rare forms are quite different from those in 21-hydroxylase deficiency. The publications cited should be consulted for details.

Adrenal Insufficiency Secondary to Anterior Pituitary Insufficiency. This condition is not usually associated with the dramatic signs and symptoms characteristic of adrenal insufficiency resulting from disease of the adrenal cortex unless there are complicating circumstances, for example, unusual fluid losses, trauma, or starvation. Hypoglycemia is the most frequent cause of symptoms. Quantita-

tion of the electrolytes in plasma often reveals a dilutional hyponatremia. The administration of 20 mg of cortisol on arising and 10 mg in late afternoon is adequate replacement therapy for most patients with anterior pituitary insufficiency. This schedule mimics, to some extent, the normal diurnal cycle of adrenal secretion. Occasional patients require additional doses. When initiating treatment, it is customary to begin cortisol first and to add thyroid replacement therapy after adrenal insufficiency is under some degree of control, on the grounds that the administration of thyroid to a hypopituitary patient may precipitate acute adrenal insufficiency. Additional treatment is necessary during periods of stress. Cortisol, 300 to 400 mg per day, should be given to approximate the normal response to severe stress.

Therapeutic Uses in Nonendocrine Diseases. Brief outlines of important uses of corticosteroids in diseases other than those involving the pituitary-adrenal complex are set forth below. The disorders discussed are not inclusive, but rather a representative list of the more common diseases for which the glucocorticoids are used.

The dosage of glucocorticoids varies greatly with the condition being treated. In the following discussion approximate doses of a representative corticosteroid congener, usually prednisone, are suggested. It is not meant to imply that prednisone has peculiar merit in general or for any particular disease over the other congeners. For comparison of doses of glucocorticoids, *see* Table 63–3.

Arthritis. In *rheumatoid arthritis*, the criterion for initiating corticosteroid therapy is progressive disease with consequent disability, despite intensive treatment with rest, physical therapy, aspirin-like drugs, gold, and other agents. The decision to embark upon a program of hormone therapy must be made with due consideration for the fact that corticosteroid therapy, once started, may have to be continued for many years or for life, with the attendant risks of serious complications. The initial dose should be small and increased slowly until the desired degree of control is attained. The symptomatic effect of small reductions should be frequently tested in order to maintain the dose as low as possible. Complete relief is not sought. A regimen of rest, physical therapy, and aspirin-like drugs is continued. The usual initial dose is about 10 mg of prednisone (or equivalent) per day in divided doses. Optimal therapy for some patients with painful symptoms confined to one or a few joints may be intra-articular injection of the steroid into the affected joints. Typical doses are 5 to 20 mg of triamcinolone acetonide or the equivalent, depending upon the size of the joint cavity.

In *osteoarthritis*, intra-articular injection of corticosteroids is recommended for treatment of episodic manifestations of acute inflammation: local heat, swelling, and pain. Injections for this purpose should be infrequent because, in both rheumatoid arthritis and osteoarthritis, a significant incidence of painless destruction of the joint, reminiscent of Charcot's arthropathy, may be associated with repeated intra-articular injections of corticosteroids.

Rheumatic Carditis. Corticosteroids are reserved for patients failing to respond to salicylates and as initial therapy for patients severely ill with fever, acute congestive heart failure, arrhythmia, and pericarditis; acute manifestations are more rapidly suppressed by corticosteroids than by salicylates, a possibly lifesaving difference in a moribund patient. A dose of approximately 40 mg of prednisone or equivalent is usually given daily, in divided amounts, although much larger doses may on occasion be required. Reactivation of the disease occurs in a number of instances following withdrawal of steroid therapy. For this reason it has been suggested that salicylates be given concurrently with corticosteroids and be continued through and after the period of withdrawal of hormone therapy.

Renal Diseases. Corticosteroids do not modify the course of acute or chronic glomerulonephritis. However, patients with some forms of the *nephrotic syndrome* attributable to systemic lupus erythematosus or to primary renal disease, except renal amyloidosis, may be benefited by corticosteroid therapy. A typical therapeutic regimen consists in the daily administration, in divided doses, of 60 mg of prednisone or equivalent (2 mg/kg of edema-free body weight in children) for 3 or 4 weeks. If a remission with a diuresis and decreased proteinuria occurs during this period, maintenance treatment is continued for as long as a year. For this, the daily dose of prednisone is given only for the first 3 days of each week (Bacon and Spencer, 1973).

Collagen Diseases. The manifestations of most of the diseases in this group are controlled by glucocorticoids. An exception is *scleroderma*, which is generally considered refractory to these agents. It is important to distinguish between scleroderma and *mixed connective tissue disease syndrome*, which is responsive to steroids (Yount *et al.*, 1973). *Polymyositis, polyarteritis nodosa,* and the granulomatous-polyarteritis group (*Wegener's granulomatosis, temporal-cranial arteritis,* and *polymyalgia rheumatica*) are treated with daily doses of prednisone, approximately 1 mg/kg or equivalent, to induce a remission. The dose is then tapered down to the minimally effective level. Glucocorticoids decrease morbidity in all these diseases and prolong the survival times of patients with polyarteritis nodosa and Wegener's granulomatosis. *In temporal (giant-cell) arteritis, adequate steroid therapy is necessary to prevent the blindness that occurs in about 20% of untreated cases. Fulminating systemic lupus erythematosus* is a life-threatening condition, the manifestations of which should be suppressed by adrenocorticosteroid therapy with doses large enough to produce a prompt effect. Treatment usually consists in a 1-mg/kg daily dose of prednisone or equivalent. Within 48 hours reduction of fever and improvement in the signs and symptoms of arthritis, pleuritis, or pericarditis should be observed. If not, the dose should be increased in 20-mg increments daily until a favorable response occurs. After the acute episode has been brought under control, corticosteroid therapy should be reduced by small steps, for example, 5 mg of prednisone per week, until signs or symp-

toms warn against further reductions. Salicylate or related drugs are then introduced and may permit a further reduction of corticosteroid dosage (Robinson, 1962). The treatment of systemic lupus erythematosus with a combination of glucocorticoids and antimetabolites, such as azathioprine, or the alkylating agent cyclophosphamide, is still experimental and not recommended for general use (Deker, 1982).

Allergic Diseases. The manifestations of allergic disease that are of limited duration, such as *hay fever, serum sickness, urticaria, contact dermatitis, drug reactions, bee stings,* and *angioneurotic edema,* can, if necessary, be suppressed by adequate doses of glucocorticoids given as a supplement to the primary therapy. It must be emphasized, however, that the effects of the steroids require some time to develop, and *severe reactions such as anaphylaxis and angioneurotic edema of the glottis require immediate therapy with epinephrine, 0.5 to 1.0 ml of a 1:1000 solution (0.5 to 1.0 mg) subcutaneously.* In life-threatening situations steroids may be given intravenously; dexamethasone sodium phosphate (8 to 12 mg or equivalent) is appropriate. In less severe diseases, such as serum sickness or hay fever, antihistaminic compounds are the drugs of first choice.

Bronchial Asthma. The corticosteroids should *not* be used routinely in the treatment of any asthmatic condition, acute or chronic, that can promptly be brought under moderate control with other measures. In *status asthmaticus,* cortisol sodium succinate (50 to 100 mg) is administered by intravenous infusion over 8 hours. The procedure is repeated daily until the acute attack is under control, following which the patient is given 10 mg of prednisone twice daily for 4 or 5 days. The dose is then reduced in steps and withdrawal planned for about the tenth day after initiation of the prednisone therapy. Under favorable circumstances, the patient can subsequently be managed once again with his prior medication.

In the treatment of *severe chronic bronchial asthma,* or *chronic obstructive pulmonary disease,* uncontrolled by other measures, the administration of a corticosteroid may be considered. The decision must be made with great care since the majority of patients, once started on corticosteroid therapy, remain indefinitely on such therapy. While some patients are effectively managed with inhalation of beclomethasone dipropionate, oral administration of prednisone in daily doses of 5 to 10 mg is required more frequently. Patients who have been taking a glucocorticoid orally must continue this medication in slowly decreasing dosage when inhalation therapy with beclomethasone is begun. Asymptomatic oropharyngeal candidiasis develops in a high percentage of patients using beclomethasone (Webb-Johnson and Andrews, 1977).

Ocular Diseases. Corticosteroids are frequently used to suppress inflammation in the eye, and employed properly they are often responsible for preservation of sight. Levine and Leopold (1973) list 28 disorders of the eye that respond to corticosteroids. They are administered locally for disease of the outer eye and anterior segment. Both natural and synthetic corticosteroids attain therapeutic concentrations in the aqueous humor following instillation into the conjunctival cul-de-sac. For disease of the posterior segment, systemic administration is required.

A typical prescription is 0.1% dexamethasone phosphate solution (ophthalmic), 2 drops in the conjunctival sac every 4 hours while awake, and 0.05% dexamethasone phosphate ointment (ophthalmic) at bedtime. For inflammations of the posterior segment of the eye, usual daily doses are approximately 30 mg of prednisone or equivalent, administered orally in divided doses.

It has been convincingly demonstrated that topical corticosteroid therapy *frequently induces intraocular hypertension* in normal eyes and further increases pressure in eyes with initially elevated pressure. The glaucoma has not always been reversible on cessation of corticosteroid treatment. It has been recommended that intraocular pressure be monitored when corticosteroids are applied to the eye for more than 2 weeks.

The local administration of corticosteroids to patients with bacterial, viral, or fungal conjunctivitis *may mask evidences of progression of the infection until sight is lost.* Corticosteroids are *contraindicated in herpes simplex* (dendritic keratitis) of the eye, because progression of the disease and irreversible clouding of the cornea may occur. Topical steroids should not be used in the treatment of mechanical lacerations and abrasions of the eye. They delay healing and promote the development and spread of infection.

Skin Diseases. The development of corticosteroid preparations suitable for topical administration has revolutionized the therapy of the more common varieties of skin disease. Maibach and Stoughton (1973) have divided 20 dermatological disorders that respond to topical corticosteroids into those that are very responsive and those that require higher concentrations of steroids, occlusion of the drug under a plastic film, or intralesional administration. Attention must be paid to the concentration of steroid used, and there are a large number of preparations of various concentrations available for topical use (Table 63–4). A typical prescription for an eczematous eruption is 1% cortisol ointment applied locally twice daily. Effectiveness is enhanced by application of the cream or ointment under a transparent plastic wrapping. Unfortunately, systemic absorption is also enhanced, occasionally sufficiently to suppress the pituitary-adrenal axis or to produce Cushing's syndrome. Adrenocorticosteroids are administered systemically for severe episodes of acute skin disorders and exacerbations of chronic disorders. The dose is usually 40 mg per day of prednisone or equivalent. Systemically administered corticosteroids may be lifesaving in *pemphigus.* Up to 120 mg of prednisone or equivalent per day may be required to control the disease.

Diseases of the Intestinal Tract. Patients severely ill with untreated *celiac sprue* can often benefit from a course of glucocorticoid therapy given at the same time that management with a gluten-free diet is begun. Prednisolone, 30 mg per day or

equivalent, is continued for 3 to 4 weeks. Patients who fail to respond to a gluten-free diet are helped by lower doses of prednisolone (7 to 12 mg per day or equivalent) for an indefinite period (Wall, 1973).

Corticosteroid therapy is indicated in selected patients with *chronic ulcerative colitis*. Mildly ill patients with bowel symptoms but without disabling systemic symptoms usually can and should be managed with rest, diet, sedation, anticholinergic agents, and chemotherapy. However, patients who do not improve should have a trial of methylprednisolone acetate, 40 mg or equivalent, in a nightly retention enema, in an attempt to induce remission. Alternate-day therapy may be effective. Severely ill patients with fever, anorexia, anemia, and malnutrition often improve dramatically when given systemic corticosteroid therapy. Large doses, 60 to 120 mg per day of prednisone, or the equivalent, are recommended. Major complications of ulcerative colitis may occur despite corticosteroid therapy. Signs and symptoms of intestinal perforation and peritonitis may be difficult to detect during corticosteroid treatment (ReMine and McIlrath, 1980).

Cerebral Edema. Corticosteroids are of value in the reduction or prevention of cerebral edema associated with neoplasms, especially those that are metastatic. In spite of widespread use of glucocorticoids for treatment of the cerebral edema due to trauma or cerebrovascular accidents, there is no convincing evidence of their value in these conditions (Nelson and Dick, 1975).

Malignancies. The chemotherapy of *acute lymphocytic leukemia* and *lymphomas* has been greatly improved by the introduction of therapy with multiple agents, and glucocorticoids are used because of their antilymphocytic effects. At the present time these diseases are treated in a complex fashion with rigidly scheduled sequences of combined drug therapy. Prednisone is commonly used in conjunction with an alkylating agent such as cyclophosphamide, an antimetabolite, and a vinca alkaloid (*see* Chapter 55).

Objective tumor regression in *carcinoma of the breast* can be induced by glucocorticoids in about 15% of patients; prednisolone (30 mg per day) has been the usual treatment. The presumed mechanism by which the corticosteroids act in these patients is through adrenocortical suppression, with an accompanying decrease in production of androgens, which are precursors of tumor-stimulating estrogens (Brennan, 1973). A beneficial response should be expected only when the tumor has estrogen and/or progesterone receptors. Other forms of therapy are usually more effective.

Diseases of the Liver. The use of glucocorticoids in the treatment of hepatic diseases has been the subject of controversy. Careful studies have now indicated several diseases of the liver in which therapy with steroids significantly improves survival rates; these are *subacute hepatic necrosis* and *chronic active hepatitis, alcoholic hepatitis,* and *nonalcoholic cirrhosis in females* (Lesesne and Fallon, 1973; Copenhagen Study Group for Liver Diseases, 1974). Only certain patients with chronic active hepatitis should receive steroid therapy. Those who benefit have symptomatic disease, histological evidence of severe disease, and a negative reaction for hepatitis B surface antigen (Berk *et al.*, 1976). Treatment of *subacute hepatic necrosis* and *chronic active hepatitis* includes prednisolone, 60 to 100 mg per day; the dose is tapered as the disease improves. Treatment of *alcoholic hepatitis* with corticosteroids is reserved for patients who are severely ill, with evidence of hepatic encephalopathy. Prednisone (40 mg per day) is given for 1 month, followed by withdrawal over a period of 2 to 4 weeks. *Nonalcoholic cirrhosis in women* should be treated with glucocorticoids if the patient does not have ascites. Daily dosages average 15 to 20 mg of prednisone or equivalent when they are adjusted to the needs of the individual patients. The data indicate that *steroid treatment lowers survival rates when ascites is present.* Treatment of cirrhotic male patients with steroids has not been shown to be beneficial.

Shock. While corticosteroids are often administered to patients in shock, there is no convincing evidence to indicate that such therapy is efficacious.

Miscellaneous Diseases. Sarcoidosis is treated with prednisone, approximately 1 mg/kg per day or equivalent, to induce a remission. Maintenance doses, which are often required for long periods of time, may be 10 mg of prednisone per day or less. In this, as in other diseases treated by prolonged steroid therapy, patients with positive tuberculin reactions or other evidence of tuberculosis should receive prophylactic antituberculosis therapy. In *thrombocytopenia,* prednisone, 0.5 mg/kg, or equivalent, is used to decrease the bleeding tendency. In severe cases and for initiation of treatment of *idiopathic thrombocytopenia,* daily doses of prednisone, 1 to 1.5 mg/kg, are employed. *Hemolytic anemias* with a positive Coombs' test are treated with prednisone, 1 mg/kg per day or equivalent. If hemolysis is severe, therapy is initiated with 100 mg of cortisol intravenously; as the disease improves, the dose is decreased. Small maintenance doses may be needed for several months if a positive response is obtained. In *organ transplantation,* high doses of prednisone (50 to 100 mg) are given at the time of the transplant surgery, usually in conjunction with immunosuppressive agents. Smaller maintenance doses (10 to 20 mg per day) are continued indefinitely, and the dosage is increased if rejection is threatened. In *aspiration of gastric contents,* prednisone (50 to 100 mg) is given for 2 to 3 days to suppress the inflammatory reaction in the lung and to prevent development of pulmonary abscess. However, corticosteroids are probably effective only if they are administered within several hours of the aspiration.

DIAGNOSTIC APPLICATIONS OF ADRENOCORTICAL STEROIDS

Potent synthetic congeners of cortisol reduce urinary excretion of cortisol metabolites by inhibition of pituitary ACTH release. The dose required is so

small, in gravimetric terms, that it contributes only negligibly to the urinary steroids. Liddle (1960) reported that the administration of 0.5 mg of dexamethasone every 6 hours for a total of eight doses results in a marked suppression of excretion of cortisol metabolites in normal persons, but does not suppress urinary steroids in individuals with Cushing's syndrome. This test is useful in distinguishing persons with some nonspecific elevation of steroid excretion, for example, that due to obesity or stress, from patients with Cushing's syndrome. The administration of 2 mg of dexamethasone every 6 hours for a total of eight doses usually causes a suppression of cortisol secretion in patients with pituitary-dependent hypercorticism, but ordinarily has little if any effect on the urinary steroids of patients with adrenal neoplasms or ACTH-producing tumors (Meador *et al.*, 1962). However, "suppressible" tumors have been reported. The results of these tests are likely to be most definite if the urinary steroids are measured daily for 2 days before and for at least 2 days during administration of the suppressing agent. Variations of this procedure (shorter test period and measurement of plasma cortisol rather than urinary metabolites) have been described (Sawin *et al.*, 1968).

INHIBITORS OF THE BIOSYNTHESIS OF ADRENOCORTICAL STEROIDS

Three pharmacological agents have proven most useful as inhibitors of adrenocortical secretion. *Mitotane* (*o,p'*-DDD), an adrenocorticolytic agent, is discussed in Chapter 55. *Metyrapone* and *aminoglutethimide* are discussed here. The subject has been reviewed by Temple and Liddle (1970).

Metyrapone. Metyrapone reduces cortisol production by inhibition of the 11β-hydroxylation reaction. Metyrapone also inhibits side chain cleavage to some degree (Cheng *et al.*, 1974), but this block is largely overcome when ACTH stimulates the gland. The biosynthetic process is terminated at 11-desoxycortisol (Figure 63–3), a compound that has practically no inhibitory influence on the secretion of ACTH. In the normal person, a compensatory increase in ACTH secretion follows, and the secretion of 11-desoxycortisol, a "17-hydroxycorticoid," is markedly accelerated. Consequently, in normal persons, administration of metyrapone induces increased plasma ACTH and renal excretion of "17-hydroxycorticoids."

Metyrapone is used to test the capacity of the pituitary to respond to a decreased concentration of plasma cortisol. A response that is greater than normal is usually found in patients with Cushing's syndrome of pituitary origin. In most cases of Cushing's syndrome due to ectopic production of ACTH there is no response to the drug. Administration of metyrapone to patients with disease of the hypothalamico-pituitary complex who are unable to achieve a compensatory increase in the rate of secretion of ACTH is, of course, not followed by increased renal excretion of 17-hydroxycorticoids.

The ability of the adrenal to respond to ACTH should be demonstrated before metyrapone is employed, for two reasons: (1) administration of metyrapone can be used as a test for normal hypothalamico-pituitary function only if the adrenal glands are capable of responding to ACTH, and (2) the drug may induce acute adrenal insufficiency in patients with reduced adrenal secretory capacity. Metyrapone also inhibits synthesis of aldosterone, which, like cortisol, is an 11β-hydroxylated compound. However, metyrapone does not typically cause a deficiency of mineralocorticoids, with a consequent loss of sodium and retention of potassium, because the inhibition of the 11β-hydroxylation reaction results in an increased production of 11-desoxycorticosterone, a mineralocorticoid.

Metyrapone has been used successfully to treat the hypercortisolism that results from adrenal neoplasms that function autonomously and from ectopic ACTH production by tumors. Its use in treatment of Cushing's syndrome resulting from hypersecretion of ACTH by the pituitary is controversial (Orth, 1978; Gold, 1979). Metyrapone has been used experimentally in patients with Cushing's syndrome during the period of time required for radiation treatment to become effective (Orth, 1978). Long-term treatment with metyrapone can cause hypertension as the result of excessive secretion of desoxycorticosterone.

Metyrapone (METOPIRONE) is 2-methyl-1,2-di-3-pyridyl-1-propanone. The drug is marketed as 250-mg oral tablets. Following two 24-hour control periods, the drug is given orally in the dose of 750 mg every 4 hours for six doses. Maximal urinary excretion of 11-desoxycorticosteroids is observed on the next day.

Aminoglutethimide. This compound, α-ethyl-*p*-aminophenyl-glutarimide, inhibits the conversion of cholesterol to 20α-hydroxycholesterol. This inhibition of the first reaction of steroidogenesis from cholesterol interrupts production of both cortisol and aldosterone.

Aminoglutethimide has been used successfully to decrease the hypersecretion of cortisol in autonomously functioning adrenal tumors and in hypersecretion resulting from ectopic production of ACTH. It has also been used in combination with metyrapone in the treatment of Cushing's syndrome that results from hypersecretion of ACTH by the pituitary (*see* Gold, 1979). Substitution of physiological doses of cortisol may be required to prevent adrenal insufficiency.

Aminoglutethimide (CYTADREN) is marketed as 250-mg oral tablets. The suggested dosage is 250 mg every 6 hours. The dose is increased by 250 mg per day at 1- or 2-week intervals until side effects prohibit further increments or until a daily dose of 2 g is achieved.

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A mechanism for the antiinflammatory effects of corticosteroids: The glucocorticoid receptor regulates leukocyte adhesion to endothelial cells and expression of endothelial–leukocyte adhesion molecule 1 and intercellular adhesion molecule 1

(neutrophils/endothelium/inflammation)

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ABSTRACT Corticosteroids are the preeminent antiinflammatory agents although the molecular mechanisms that impart their efficacy have not been defined. The endothelium plays a critical role in inflammation by directing circulating leukocytes into extravascular tissues by expressing adhesive molecules for leukocytes [e.g., endothelial–leukocyte adhesion molecule 1 (ELAM-1) and intercellular adhesion molecule 1 (ICAM-1)]. We therefore determined whether corticosteroids suppress inflammation by inhibiting endothelial expression of adhesion molecules for neutrophils (polymorphonuclear leukocytes). Preincubation of endothelial cells with endotoxin [lipopolysaccharide (LPS), 1 μg/ml] led to a 4-fold increase in subsequent adherence of polymorphonuclear leukocytes ($P < 0.0001$, $n = 10$) to endothelial cells, an increase that was markedly attenuated when endothelial cells were treated with dexamethasone ($IC_{50} < 1$ nM, $P < 0.0001$, $n = 6$ or 7) during preincubation with LPS. Moreover, the steroid receptor agonist cortisol (10 μM), but not its inactive metabolite tetrahydrocortisol (10 μM), diminished LPS-induced endothelial cell adhesiveness. Further evidence that the action of dexamethasone was mediated through ligation of corticosteroid receptors [human glucocorticoid receptors (hGRs)] was provided by experiments utilizing the steroid antagonist RU-486. RU-486 (10 μM), which prevents translocation of ligated hGR to the nucleus by inhibiting dissociation of hGR from heat shock protein 90, completely aborted the effect of dexamethasone on adhesiveness of endothelial cells ($P < 0.0005$, $n = 3$). Treatment of endothelial cells with LPS (1 μg/ml) stimulated transcription of ELAM-1, as shown by Northern blot analysis, and expression of membrane-associated ELAM-1 and ICAM-1, as shown by quantitative immunofluorescence (both $P < 0.001$, $n = 9$). Dexamethasone markedly inhibited LPS-stimulated accumulation of mRNA for ELAM-1 and expression of ELAM-1 and ICAM-1 ($IC_{50} < 10$ nM, both $P < 0.001$, $n = 4$–9); inhibition of expression by dexamethasone was reversed by RU-486 (both $P < 0.005$, $n = 4$–6). As in the adhesion studies, cortisol but not tetrahydrocortisol inhibited expression of ELAM-1 and ICAM-1 (both $P < 0.005$, $n = 3$ or 4). In contrast, sodium salicylate (1 mM) inhibited neither adhesion nor expression of these adhesion molecules. These studies suggest that antagonism by dexamethasone of endotoxin-induced inflammation is a specific instance of the general biological principle that the glucocorticoid receptor is a hormone-dependent regulator of transcription.

Although glucocorticoids are among the most potent and widely used antiinflammatory agents, the mechanisms by which they reduce inflammation are unknown. Various hypotheses have been proposed; these include "allosteric" effects on enzymes (1), redirection of lymphocyte traffic (2), direct inhibition of various phospholipases (3), induction of such proteins as lipocortin (4), inhibition of the transcription of various cytokines and metalloproteases (5–14), and our own earlier suggestion that glucocorticoids stabilize lysosomal and other cellular membranes (15, 16). However, none of these hypotheses is sufficient to account for the well-known pharmacologic effects of glucocorticoids in humans: leukocytosis (17), inhibition of leukocyte recruitment to inflamed areas (18, 19), retention of lymphocytes in the lymphatic circulation with shrinkage of peripheral lymph nodes, and the promotion of microbial infection (2).

Recent studies have suggested that endothelial cells can direct the traffic of leukocytes into inflamed and infected areas (heterotypic adhesion) via the regulated expression of surface adhesive molecules [e.g., GMP140, endothelial–leukocyte adhesion molecule 1 (ELAM-1), intercellular adhesion molecule 1 (ICAM-1), and vascular cell adhesion molecule (VCAM) (20–23)]. In a complementary fashion, leukocytes also express proteins [CD11a-c/CD18, L-selectin, or lectin/epidermal growth factor cell adhesion molecule 1 (LECAM-1)] on their surface that mediate their specific localization to sites of inflammation (24, 25). Agents that modulate the interaction of leukocytes with the endothelium may, therefore, possess potent antiinflammatory properties.

We now present data compatible with the hypothesis that glucocorticoids—at nanomolar concentrations—inhibit the expression of adhesive molecules ELAM-1 and ICAM-1 by endotoxin-activated endothelial cells and thereby interfere with the traffic of leukocytes into inflamed areas. Pretreatment of endothelial cells with corticosteroids prevents endothelial cells from becoming more adhesive for neutrophils [polymorphonuclear leukocytes (PMNs)] and diminishes stimulated expression of ICAM-1 and ELAM-1, molecules critical for neutrophil adhesion. Moreover, these data make it likely that corticosteroids regulate ELAM-1 at the transcriptional level.

MATERIALS AND METHODS

Materials. Lipopolysaccharide (LPS, *Salmonella typhimurium*) was obtained from Calbiochem and *N*-formylmethionylleucylphenylalanine was obtained from Vega Biochemi-

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Abbreviations: ELAM-1, endothelial–leukocyte adhesion molecule 1; ICAM-1, intercellular adhesion molecule 1; PMN, polymorphonuclear leukocyte; LPS, lipopolysaccharide; HUVEC, human umbilical vein endothelial cell; IL, interleukin; TNF, tumor necrosis factor; hGR, human glucocorticoid receptor; FITC, fluorescein isothiocyanate.

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cal. Collagenase, dexamethasone, hydrocortisone, tetrahydrocortisol, and sodium salicylate were obtained from Sigma. RU-486 was a gift to Herbert Samuels (Roussel-Uclaf). Medium 199, RPMI 1640, and fetal bovine serum were obtained from GIBCO. Ficoll/Hypaque was purchased from Nyegaard (Oslo). All other salts and reagents were of the highest quality that could be obtained.

Monoclonal Antibodies. In these studies the monoclonal antibodies used included antibodies directed against ICAM-1 (84H10, AMAC, Westbrook, ME), ELAM-1 (BMA 4D10, Accurate Chemicals, Westbury, NY, and H18/7, a generous gift of Michael Bevilacqua, San Diego), MOPC21, and UPC10, nonbinding isotype controls. Fluorescein-labeled anti-IgG, goat anti-mouse fluorescein isothiocyanate (GAM-FITC) was obtained from Coulter Immunology. Endothelial cells were also stained with rhodamine-labeled *Ulex europaeus* agglutinin I (Vector Laboratories); all monoclonal antibody studies were performed in ice-cold phosphate-buffered saline (PBS) with 0.02% sodium azide and 0.025% bovine serum albumin.

Culture of Endothelial Cells. Human umbilical vein endothelial cells (HUVECs) were cultured as described (26). All experiments were performed on endothelial cells in their third passage.

Incubation of Endothelial Cells with Pharmacologic Agents. Endothelial cells were stimulated by incubation with LPS (1 μg/ml) in a medium consisting of RPMI 1640/10% fetal bovine serum for 4 hr at 37°C in a 5% CO_2 atmosphere with or without other agents as indicated. The monolayers were then washed three times.

PMN Adherence to Endothelial Monolayers (Heterotypic Adherence). PMNs (150,000 per well), isolated from whole blood as described (27), suspended in RPMI 1640 medium, were added to monolayers of endothelial cells and incubated for 10 min at 37°C in a 5% CO_2 atmosphere and adherence was determined as described (28). Pretreatment of unstimulated HUVECs with each of the agents used did not affect basal PMN adherence (data not shown). The highest concentration of diluent used (ethanol, 0.1%) also had no effect on PMN adherence to LPS-stimulated endothelium.

In some experiments PMNs were labeled with ^{111}In and after incubation of labeled PMNs with endothelium for 10 min at 37°C adherence was determined by a previously described method (29) and expressed as % adherence.

Expression of ELAM-1 and ICAM-1. After incubation for 4 hr with various stimuli and agents the HUVECs were removed from wells by exposure to EDTA (0.01%, wt/vol) in PBS for 10 min at 37°C in a 5% CO_2 atmosphere followed by gentle scraping with a rubber policeman and trituration. Cells were resuspended in ice-cold saline containing sodium azide (0.02%) and saturating concentrations of antibodies, incubated for 30 min at 4°C, washed and counterstained with fluorescein-labeled anti-IgG for 30 min at 4°C, washed, and fixed in formaldehyde (3.7% in PBS). HUVECs were then analyzed with a FACScan (Becton Dickinson) (30). In these experiments the fluorescence of cells stained with an isotype control antibody (MOPC21) was 33 ± 13.

Analysis of Message for ELAM-1. HUVECs were incubated without or with stimuli in the presence and absence of dexamethasone (0.1 μM) for 3 hr at 37°C. Following treatment with collagenase/EDTA [0.1%/0.01% (wt/vol) 10 min at 37°C] cells were suspended and washed, and mRNA was isolated by use of the FastTrack kit following the instructions provided (Invitrogen, San Diego). After electrophoresis through agarose the mRNA was transferred to nitrocellulose and hybridized with full-length ^{32}P-labeled cDNA probes for ELAM-1 (generously supplied by Tucker Collins) and actin (Stratagene) under conditions of high stringency. Labeled cDNA for ELAM-1 hybridized with a single band of 3.65 kilobases (kb) and labeled cDNA for actin hybridized with a single band of 2.0 kb. Autoradiograms were prepared and then analyzed by laser densitometry (31).

Statistical Analysis. The significance of differences among and between experimental treatment groups was determined by means of the appropriate level of analysis of variance and determination of separate post-hoc variances by means of the CSS Software (Statsoft, Tulsa, OK) using an IBM-compatible computer.

RESULTS

Glucocorticoids Prevent Adhesion of PMNs to LPS-Stimulated Endothelial Cells (Heterotypic Adhesion). Activation of HUVECs with LPS or cytokines [interleukin 1 (IL-1) and tumor necrosis factor α (TNF-α)] causes endothelial cells to bind unstimulated PMNs more avidly (20, 21, 30, 32). When we treated HUVECs with LPS (1 μg/ml) we found, as expected, that ≈4-fold more PMNs adhered to treated than untreated HUVECs (50 ± 3 vs. 13 ± 2 PMNs per high-power field, $P < 0.0001$). Dexamethasone inhibited PMN adhesion to the LPS-stimulated HUVECs in a dose-dependent manner ($IC_{50} < 1$ nM, $P < 0.005$, Fig. 1).

Glucocorticoid Receptors Mediate the Effects of Glucocorticoids on Adhesion of PMNs to HUVECs. To determine whether the modulation of endothelial adhesiveness by dexamethasone was receptor-mediated we studied the effects of RU-486, a noncompetitive antagonist of glucocorticoid receptors (33, 34). RU-486 (10 μM) completely reversed the effect of dexamethasone on LPS-stimulated adhesiveness of HUVECs (Fig. 1, $P < 0.0005$). In other experiments 100-fold higher concentrations of the less potent steroid receptor agonist cortisol (IC_{50} = 100 nM, n = 2), but not its inactive metabolite tetrahydrocortisol (0.1 and 10 μM), diminished the LPS-stimulated increment in adhesiveness (data not shown). Further, neither indomethacin (10 μM) nor sodium salicylate (1 mM) affected the LPS-stimulated increment in endothelial adhesiveness.

Glucocorticoids Modulate Expression of ELAM-1 and ICAM-1 on LPS-Stimulated Endothelium. We next determined whether the effect of corticosteroids on endothelial cell adhesiveness resulted from diminished expression of ELAM-1 or ICAM-1. After stimulation by LPS, HUVECs increased expression of ELAM-1 by 321% ± 68% (SEM, $P < 0.0001$, n = 16) and ICAM-1 by 250% ± 38% ($P < 0.0001$, n = 16, Fig. 2).

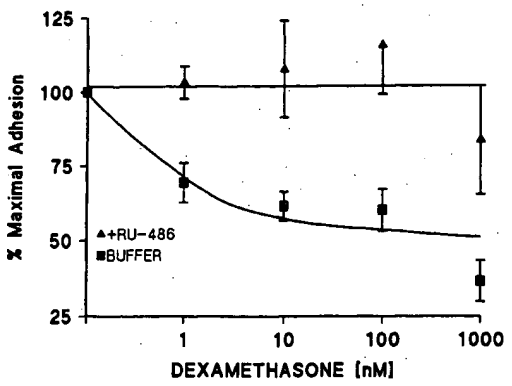

FIG. 1. Inhibition by dexamethasone (0.1–1000 nM) of PMN adhesion to LPS-stimulated HUVECs. HUVECs were stimulated, as above, in the presence or absence of various doses of dexamethasone and RU-486 (10 μM). Adherence of PMNs to unstimulated endothelium was determined and subtracted from the PMN adherence to stimulated endothelium and then expressed as a percentage of net adherence of PMNs to untreated endothelial monolayers. In the absence of dexamethasone RU-486 did not affect adhesion of PMNs to either unstimulated (122% ± 16% of control, n = 4) or LPS-treated endothelium (114% ± 11% of control, n = 4).

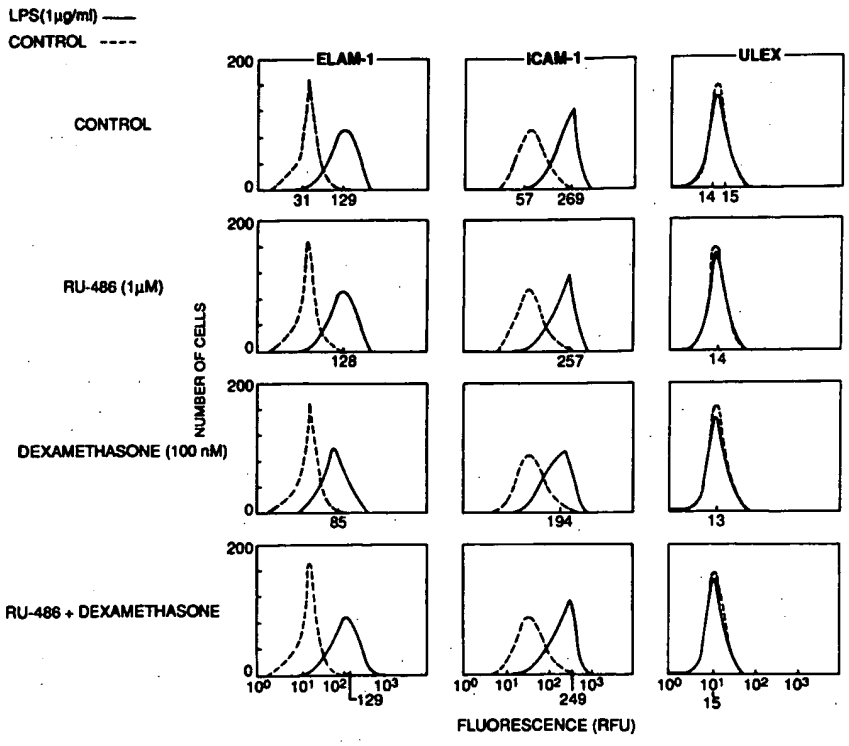

FIG. 2. Inhibition by dexamethasone of the increased surface expression of ICAM-1 and ELAM-1 by LPS-stimulated HUVECs. Fluorescence histograms are as follows: top row, resting and activated (LPS, 1 μg/ml) HUVECs stained with antibodies directed against ICAM-1, ELAM-1, and *Ulex europaeus* agglutinin I; second row, resting and activated HUVECs activated in the presence of RU-486 (10 μM); third row, HUVECs activated in the presence of dexamethasone (0.1 μM); and bottom row, resting and activated HUVECs activated in the presence of dexamethasone (0.1 μM) and RU-486 (10 μM). Mean fluorescence of cells stained with FITC-labeled antibody (MOPC-21) alone was 10 relative fluorescence units (RFU). Shown is a representative experiment of seven, the mean results of which are presented in Figs. 3 and 4.

Dexamethasone (IC_{50} < 1 nM) inhibited the LPS-stimulated expression of ELAM-1 and ICAM-1 ($P < 0.00001$ and $P < 0.00001$, respectively, Fig. 3) without altering basal expression of these molecules (data not shown). Dexamethasone did not alter binding of rhodamine-labeled *Ulex europaeus* agglutinin I to the endothelial cell surface (Fig. 2). Similarly, none of the compounds studied affected the nonspecific binding of antibodies MOPC21, UPC10 (isotype controls), or FITC anti-mouse IgG (data not shown). As expected, RU-486 completely reversed the effect of dexamethasone on the LPS-stimulated expression of ICAM-1 and ELAM-1 ($P < 0.004$, Figs. 2 and 4).

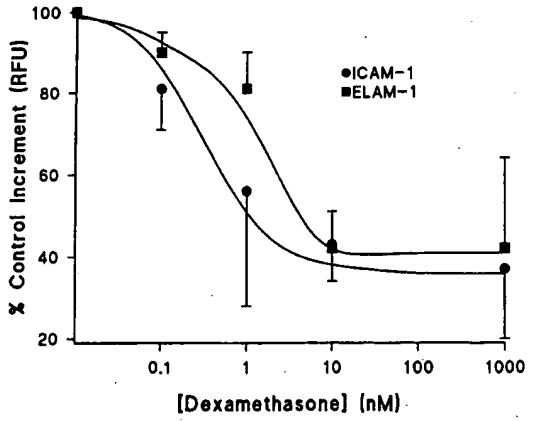

FIG. 3. Inhibition by dexamethasone (0.1 nM–10 μM) of the up-regulation of ICAM-1 and ELAM-1 expression on LPS-stimulated HUVECs. The results shown represent the means ± SEM of 4–10 experiments. RFU, relative fluorescence units.

Although RU-486 appeared to diminish LPS-stimulated up-regulation of ICAM-1 (Fig. 4), the difference observed was not statistically significant. Similarly, cortisol, but neither tetrahydrocortisol nor sodium salicylate, inhibited expression of ICAM-1 and ELAM-1 ($P < 0.003$, Fig. 5). None of the agents studied affected binding of *Ulex europaeus* agglutinin I to HUVECs (Fig. 2).

Glucocorticoids Prevent Accumulation of Message for ELAM-1 in Response to LPS and IL-1α But Not in Response to TNF. To further define the mechanism by which glucocorticoids inhibit up-regulation of adhesive molecules, we studied the effect of dexamethasone (0.1 μM) on the level of mRNA for ELAM-1 in HUVECs. Treatment of HUVECs with LPS, IL-1α (20 units/ml), and TNF (50 units/ml) stimulated a marked increase in detectable message for ELAM-1 in HUVECs (Fig. 5). Dexamethasone (0.1 μM) did not affect basal levels of mRNA for ELAM-1 but markedly inhibited the LPS- and IL-1α-stimulated increase in message. In contrast, dexamethasone did not affect the TNF-stimulated increment in detectable ELAM-1 message, an observation that suggests that glucocorticoids do not directly affect stability of message for ELAM-1. Although these findings need to be fortified by further studies of the effects of glucocorticoids on the stability of ELAM-1 message and the rate of transcription of ELAM-1 by stimulated endothelium, the results suggest that glucocorticoids act at the transcriptional level.

Dexamethasone Does Not Reverse the Effect of TNF-α on Endothelial Adhesiveness for PMNs. Since dexamethasone did not affect the level of message for ELAM-1 in TNF-α-stimulated HUVECs, we sought to determine whether this was reflected in the adhesiveness of TNF-α-treated endothelium for PMNs. As previously reported, TNF-α (50 units/ml)

account for the inhibition of ELAM-1 expression by dexamethasone. Steroid–receptor complexes also participate in protein–protein interactions with *jun*, preventing its interaction at AP-1 regulatory sites of the 5′ flanking regions of appropriate genes (36, 37), although the AP-1 site present in the gene for ELAM-1 does not appear to participate in the regulation of ELAM-1 (41).

Our results appear to differ from those of Bochner *et al.* (5), who found that prolonged (24 hr) treatment of microvascular endothelium from foreskin with glucocorticoids did not prevent LPS from modulating PMN adhesion, whereas, as we report here, treatment of HUVECs for 4 hr dramatically diminished adhesiveness. The inefficacy of prolonged treatment with corticosteroids may have been due to "desensitization" of hGR (i.e., complete depletion of hGR in the cytosol) in the presence of high concentrations of agonist (42). Alternatively, occupancy of hGR may only transiently transactivate genes that regulate ELAM-1 transcription or translation, permitting subsequent activation of ELAM-1 transcription by LPS.

We were surprised to observe that dexamethasone did not inhibit accumulation of mRNA for ELAM-1 induced by TNF-α. In parallel studies Ghezzi and Sipe (43) found that dexamethasone inhibited LPS-stimulated, but not TNF- or IL-1-stimulated, serum amyloid protein A secretion in mice. Thus LPS, TNF, and IL-1 may induce the transcription of ELAM-1 by several different mechanisms, only some of which are sensitive to corticosteroids.

These experiments permit us to suggest a mechanism for the antiinflammatory effects of glucocorticoids: acting via their receptor, glucocorticoids prevent the recruitment of leukocytes at inflammatory loci by inhibiting the display of adhesive molecules on the surface of the endothelium. This hypothesis rests on three separate lines of evidence: *functional*, at nanomolar concentrations glucocorticoids inhibit endotoxin-stimulated increases of endothelial adhesiveness for leukocytes; *phenotypic*, at similar concentrations they inhibit the stimulated expression of adhesive molecules on the surface of HUVECs; *genotypic*, glucocorticoids inhibit accumulation of mRNA for ELAM-1 in endotoxin- and IL-1-stimulated cells. In addition to these direct receptor-mediated effects of glucocorticoids on the capacity of HUVECs to localize the inflammatory response, glucocorticoids also inhibit the ligand-induced release of cytokines (IL-1, IL-3, and TNF-α) by endothelial and other inflammatory cells (5–12). These findings suggest the general hypothesis that corticosteroids act as antiinflammatory agents by diminishing, directly and indirectly, the ability of HUVECs to direct leukocyte traffic into inflamed or infected tissue. Our data also provide a reasonable explanation for the opposing effects of endotoxin and glucocorticoids in infection and immunity (15, 16).

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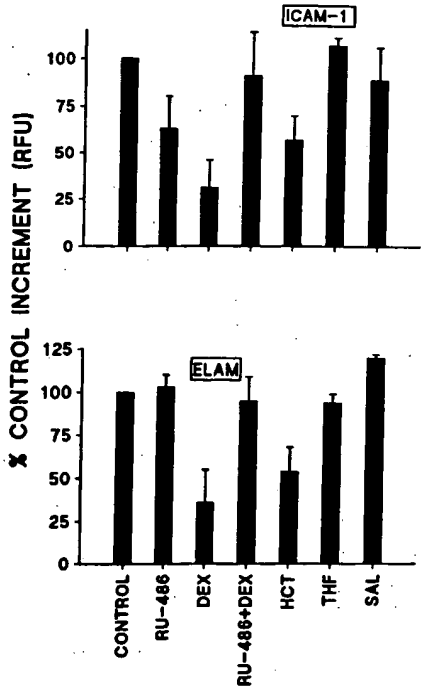

FIG. 4. Corticosteroids inhibit the LPS-stimulated expression of ICAM-1 and ELAM-1. HUVECs were incubated with LPS (1 μg/ml) in the presence of dexamethasone (DEX, 100 nM), RU-486 (10 μM), dexamethasone plus RU-486, cortisol (HCT, 10 μM), tetrahydrocortisol (THF, 10 μM), or sodium salicylate (SAL, 1.25 mM). (*Upper*) Effect of the various compounds tested on the LPS-induced increment in expression of ICAM-1 expressed as a percentage of the increment induced by LPS alone. (*Lower*) Effect of these same compounds on the LPS-stimulated increment in ELAM-1 expression. Shown are the means ± SEM of 3–10 experiments. RFU, relative fluorescence units.

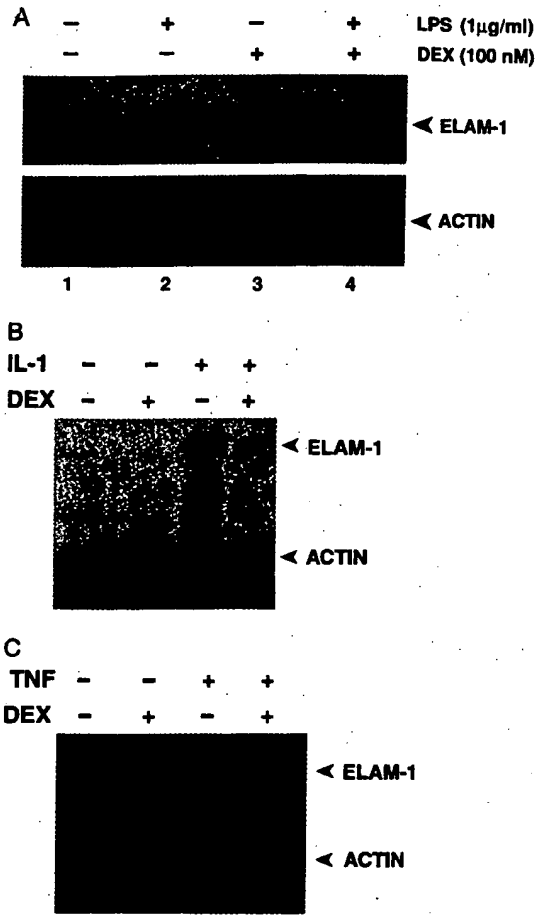

FIG. 5. Dexamethasone inhibits the stimulated increase in mRNA for ELAM. (*A*) HUVECs were incubated without (−) or with (+) LPS (1 μg/ml) in the presence (+) and absence (−) of dexamethasone (DEX, 0.1 μM). When normalized for the content of actin mRNA, LPS stimulated a 234% increase in mRNA for ELAM. Dexamethasone did not affect the basal level of mRNA for ELAM-1 (98% of control) but completely inhibited the LPS-stimulated increase in mRNA for ELAM-1 (90% inhibition). Similar results were found in a second experiment. (*B*) HUVECs were incubated with (+) or without (−) IL-1α (20 units/ml) in the presence (+) or absence (−) of dexamethasone (0.1 μM). When normalized to the content of actin mRNA, dexamethasone inhibited the IL-1α-induced increase in mRNA for ELAM-1 by 95% without affecting basal levels of mRNA for ELAM-1 (104% of control). Similar results were found in a second experiment. (*C*) HUVECs were incubated with (+) or without (−) TNF-α (50 units/ml) in the presence (+) or absence (−) of dexamethasone (0.1 μM). When normalized to the content of actin mRNA, dexamethasone did not affect either basal mRNA for ELAM-1 (97% of control) or the TNF-α-induced increment in mRNA for ELAM-1 (110% of control). Similar results were found in a second experiment.

rendered the endothelium more adhesive to PMNs (23% ± 1% adherence vs. 9% ± 2% adherence, $n = 4$, $P < 0.01$) and dexamethasone did not diminish the increased adhesiveness of TNF-stimulated endothelium for PMNs (21% ± 1% adherence, $n = 4$).

DISCUSSION

We show here that one important mechanism by which glucocorticoids may affect the inflammatory response is modulation of the capacity of the endothelium to respond to an inflammatory stimulus. Glucocorticoids, acting at their cytoplasmic receptors, diminish the LPS-stimulated increase in endothelial adhesiveness for resting PMNs and diminish transcription and expression of pro-inflammatory adhesive molecules on the surface of the endothelium. Since recent studies have increasingly pointed to the central role of the endothelium in directing the traffic of leukocytes into inflamed areas, our observations bear directly on the mechanism for the antiinflammatory effects of glucocorticoids. Moreover, our results help to explain the dramatic leukocytosis observed in patients taking therapeutic doses of corticosteroids [concentrations similar to those studied here (17)].

RU-486 stabilizes the association of steroid receptors with heat shock protein 90 in the presence of ligand, which prevents translocation of glucocorticoid receptors to the nucleus and thereby blocks transcription of genes containing glucocorticoid-responsive elements (33, 35–40). Our demonstration that RU-486 reverses the effects of dexamethasone on the adhesive qualities of HUVECs and the expression of adhesive molecules on their surface is therefore most consistent with the hypothesis that the effects of steroids on the endothelium are mediated through glucocorticoid receptors. Further confirmation is provided by the observation that cortisol, but not its inactive metabolite tetrahydrocortisol, inhibits endothelial cell responses to LPS. The absence of a glucocorticoid-responsive element in the gene for ELAM-1 suggests that glucocorticoids must either interfere directly with a transcriptional regulator of ELAM-1 transcription or induce the synthesis of a second regulatory element. Montgomery and co-workers (41) have demonstrated that NFκB regulatory elements are necessary (but not sufficient) for transcription of ELAM-1. Thus the induction by human glucocorticoid receptor (hGR) of the synthesis of a counterregulatory element such as IκB could

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Immunology

Class II major histocompatibility complex molecules of murine dendritic cells: Synthesis, sialylation of invariant chain, and antigen processing capacity are down-regulated upon culture

(epidermal Langerhans cells/dendritic cell differentiation/T-cell sensitization)

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ABSTRACT **Dendritic cells (DCs), such as Langerhans cells (LCs) of the epidermis and the DCs of lymphoid organs such as spleen, are potent antigen presenting cells. DCs express high levels of major histocompatibility complex (MHC) class II molecules, but, partly because of the low numbers of primary DCs in any tissue, there has been no detailed study of the biochemistry of their class II molecules. This information may be needed to help explain recent findings that DCs process native protein antigens when freshly isolated from epidermis and spleen. Processing ceases during culture, yet a strong accessory function for activating resting T cells develops. We studied immunoprecipitates of DC class II and invariant chain (I_i) molecules by two-dimensional gel electrophoresis. We found that (*i*) freshly isolated LCs synthesize large amounts of class II and I_i polypeptides; (*ii*) I_i molecules that are known to be involved in antigen processing display an unusually large number of sialic acids in fresh LCs; (*iii*) with culture, class II and I_i synthesis decreases dramatically and has virtually ceased at 3 days; and (*iv*) the turnover of class II in pulse/chase experiments is slow, being undetectable over a 12- to 32-hr culture period, whereas the turnover of I_i is rapid. We conclude that MHC class II molecules of DCs do not seem to be qualitatively unique. However, the regulation of class II and I_i expression is distinctive in that biosynthesis proceeds vigorously for a short period of time and the newly synthesized class II remains stably on the cell surface, whereas I_i turns over rapidly. This may enable DCs to process and retain antigens in the peripheral tissues such as skin and migrate to the lymphoid organs to activate T cells there.**

Dendritic cells (DCs) represent a system of abundantly major histocompatibility complex (MHC) class II-expressing leukocytes. They occur in nonlymphoid organs, blood, afferent lymph, and lymphoid tissues (1). Two states of differentiation can be distinguished (2, 3). "Immature" DCs, exemplified by freshly isolated epidermal Langerhans cells (LCs), are weak stimulators of resting T cells both in the allogeneic mixed leukocyte reaction (4, 5) and in polyclonal responses such as oxidative mitogenesis, concanavalin A mitogenesis, and anti-CD3 mitogenesis (6). These fresh LCs (7, 8) as well as fresh DCs from spleen (9), however, are efficient in processing native protein antigens for MHC class II-restricted presentation to presensitized peptide-specific T cells (*in vivo*-primed T cells, clones, T–T hybridomas). Upon 1–3 days of culture in macrophage- or keratinocyte-conditioned medium, or in culture medium supplemented with granulocyte/macrophage colony-stimulating factor, their functional properties become inverted. "Mature" DCs lose the capacity to process exogenous antigens but at the same time acquire the ability to sensitize resting T cells (2, 3). The loss of processing function is paralleled by the loss of acidic organelles such as endosomes (10), which are known as the compartments where class II encounters processed antigen (11–13).

Since MHC class II molecules are the essential elements of antigen processing and presentation, we studied in detail the composition and the chemical nature of class II polypeptides and associated invariant chains (I_i) in the course of DC maturation/differentiation in culture.

MATERIALS AND METHODS

Mice. BALB/c mice were purchased from Charles River Wiga, Sulzfeld, F.R.G., and were used between 6 and 10 weeks of age.

Cells. Fresh [day 0 LCs (LCd0)] and cultured [day 1 and day 3 LCs (LCd1 and LCd3)] LCs were prepared and enriched from ear skin as described (4, 14, 15). DCs were obtained from spleen (16). Thioglycollate-elicited peritoneal macrophages were induced to express MHC class II by recombinant murine interferon γ (Stratech, London) (17). In addition, a macrophage line (P388D1) and a pre-B-cell line (18-81) were used.

Immunolabeling Procedures. Epidermal sheets were prepared as described (18). Single cell suspensions were attached to multiwell microscopic slides coated with poly-L-lysine (50 μg/ml; type VII; Sigma). Cells were briefly fixed with 4% paraformaldehyde. Antibody and washing solutions contained 0.02% saponin (Sigma) to enhance penetration. Monoclonal antibody (mAb) In1 (19), rat IgG2b anti-murine invariant (I_i) chain, reacting with intracytoplasmic I_i31 and I_i41 chains (20) was used. Binding of In1 was visualized with biotinylated anti-rat immunoglobulin (not cross-reactive with mouse immunoglobulin; Vector, Burlingame, CA) followed by streptavidin fluorescein isothiocyanate (Amersham). To identify LCs, this incubation sequence was extended by rat immunoglobulin to block free anti-rat binding sites, and tetramethylrhodamine B isothiocyanate-conjugated anti-I-$A^{b,d}$ (B21-2; TIB229 from the American Type Culture Collection).

Metabolic Labeling. Cells were washed three times in methionine-free RPMI 1640 medium (GIBCO). Cells (1×10^6) were resuspended in 60 μl of methionine-free RPMI 1640 medium and 100 μCi (1 Ci = 37 GBq) of [^{35}S]methionine (SJ204; Amersham) was added. With partially enriched

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Abbreviations: EC, epidermal cell; DC, dendritic cell; I_i, invariant chain; LC, epidermal Langerhans cell; LCd0, freshly isolated LC; LCd1, 1-day cultured LC; LCd3, 3-day cultured LC; MHC, major histocompatibility complex; mAb, monoclonal antibody.

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LCd0, up to 40 × 10^6 total epidermal cells (ECs) were incubated in proportional volumes and radioactivities. After 90–120 min at 37°C, the tubes were filled up with 15 ml of warm culture medium and incubated for another hour. Then cells were washed and lysed. For pulse/chase experiments, 30 × 10^6 ECs (containing 6 × 10^6 LCd0) and 30 × 10^6 macrophages were labeled with 40 μCi per 1 × 10^6 ECs in 200 μl for 15 min at 37°C. After three washes in complete medium, ECs were cultured as described above for 12 and 32 hr. Macrophages (15 × 10^6) were cultured for the same periods in Teflon beakers.

Cell-Surface Iodination. Cell-surface proteins were labeled by the lactoperoxidase method (21).

Immunoprecipitation and Two-Dimensional Gel Electrophoresis. This was done as described in detail previously (22), using mAb In1 (see above), rat mAb 17/227, anti-I-A^d/E^d, and rat mAb NLDC-145, anti-DC-specific antigen (23). On two-dimensional gels, proteins were separated by charge using nonequilibrium pH gradient gel electrophoresis in the first (horizontal) dimension and by size using reducing NaDodSO$_4$/PAGE in the second (vertical) dimension. To achieve a semiquantitative comparison between the various cell populations, equal numbers of class II-expressing cells were labeled and immunoprecipitated and gels were scanned by densitometry (Laser Densitometer, Molecular Dynamics, Sunnyvale, CA).

Functional Assays. Oxidative mitogenesis assays (4, 14) and antigen-specific hybridoma assays (8, 10) were done as described.

RESULTS

Functional Properties of Immature and Mature DCs. Freshly isolated LCs are weak stimulators of resting T cells (4) but excellent processors of native protein as determined with peptide-specific T-cell clones (7) or hybridomas (refs. 8 and 10; Table 1). After 2–3 days of culture, LCs selectively lose processing capacity; in the same time, however, they become much more efficient in stimulating resting T cells (Table 1).

Expression of MHC Class II Molecules and Associated I_i on DCs. It has been shown that immature DCs—i.e., resident LCs—express substantial levels of MHC class II antigens (4, 16, 24). We inspected epidermal sheets by immunohistochemistry for the presence of class II and associated I_i. Both molecules are coexpressed in LCs but are not detectable in surrounding keratinocytes (Fig. 1 *A* and *B*).

Upon culture of LCs, the surface expression of MHC class II molecules is rapidly (i.e., within hours) increased up to 10-fold (4, 24, 25) and resembles class II expression on splenic and thymic DCs. In contrast, the expression of intracellular I_i chain decreased upon 3-day culture of LCs as visualized with mAb In1 (Fig. 1 *C–H*; ref. 8). Equally low amounts of cytoplasmic I_i were immunostained in spleen DCs (data not shown).

High Rate of I_i and Class II Biosynthesis in Immature DCs and Its Decrease upon Their Maturation in Culture. Although class II and I_i genes are located on different chromosomes and their structural genes are not related under immune-activated conditions, their expression is tightly co-regulated (26). Two-dimensional separation of metabolically labeled class II and I_i immunoprecipitates reveals that freshly isolated LCs synthesize large amounts of class II and I_i polypeptides (Fig. 2 *A* and *F*). When LCs were cultured for different times and their biosynthesis was analyzed, profound changes were observed. The rate of synthesis of both class II and I_i molecules dropped sharply (Fig. 2 *B–D*; ref. 8). After 1 day of culture, this became evident and within 3 days LCs had virtually stopped class II and I_i production. Spleen DCs synthesize considerably less class II and I_i than fresh LCs, albeit not as little as LCd3 (Fig. 2*E*). The down-regulation of class II/I_i production in cultured LCs was selective, because the same cells actively synthesized another protein—namely, the DC antigen immunoprecipitated by mAb NLDC-145 (8, 23) (data not shown).

Unusual Expression and Maturation of I_i in Immature LCs. The I_i gene encodes two polypeptides—I_i31 and I_i41 (27). In B cells, I_i41 usually represents 10% or less of I_i (19, 28) (Fig. 3*E*). In freshly isolated LCs, however, the proportion of I_i41 in relation to I_i31 is unusually high (Fig. 3*A*). Densitometric analysis of the gels depicted in Fig. 3 gave I_i31/I_i41 ratios of 1.3 (43% I_i41) for fresh LCs, 3.4 (23% I_i41) for spleen DCs, 2.3 (30% I_i41) for macrophages, and 10.4 (9% I_i41) for B cells.

Upon intracellular transport of class II and I_i polypeptides to trans-Golgi compartments, their glycan side chains are sialylated (29). I_i chains from freshly isolated LCs acquire an unusual number of sialic acids (Fig. 3*A*; up to 17 acidic spots), which increase the molecular weight of I_i31 up to 45,000. Similarly, I_i41 is intensely sialylated. As a consequence of sialylation, some I_i chains are more acidic than class II α chains (Fig. 3*A*, far right). Upon maturation of LCs, the

Table 1. Reciprocal expression of antigen processing and T-cell sensitizing capacities in fresh and cultured LCs

Antigen presenting cells	Dose of antigen presenting cells						
	10^4	3 × 10^3	10^3	3 × 10^2	10^2	3 × 10^1	10
Hybridoma assay	Proliferation of CTLL-2						
A. fLCs −	6.2	5.4	5.9	5.1	5.0	5.4	5.2
B. fLCs + myo	129.3	172.9	199.3	145.6	121.1	52.1	17.8
C. cLCs −	1.0	1.5	2.5	1.3	2.8	2.1	2.1
D. cLCs + myo	2.7	1.0	2.0	2.4	2.3	1.9	1.6
Oxidative mitogenesis	Proliferation of T cells						
E. 0-hr LCs	—	44.9	15.5	5.9	2.7	—	—
F. 12-hr LCs	—	45.9	19.3	6.5	2.2	—	—
G. 32-hr LCs	—	180.4	144.0	52.8	15.6	—	—
H. 12-hr MAs	—	8.6	4.2	2.1	1.6	—	—
I. 32-hr MAs	—	8.7	4.9	3.0	2.4	—	—

The hybridoma assay was as follows. Antigen processing capacity was determined by measuring interleukin 2 production of myoglobin-peptide-specific hybridoma 11.3.7 by means of CTLL-2 indicator cells (rows A–D). Note that freshly isolated LCs (fLCs) efficiently activate the hybridoma in the presence of sperm whale myoglobin protein (myo) (row B). Cultured LCs (cLCs) have lost this capacity (row D). Oxidative mitogenesis was as follows. LCs and peritoneal macrophages (MAs) cultured for 0, 12, and 32 hr were used to stimulate periodate-modified resting T cells. Note that 12-hr LCs (row F), which express levels of class II equivalent to those of 32-hr LCs (row G) (5, 24), are as weak stimulators as are freshly isolated LCs (row E). Class II-positive macrophages do not activate resting T cells (rows H and I). This experiment was done in parallel to the labeling experiment in Figs. 4 and 5. Results are expressed as cpm × 10^{-3}.

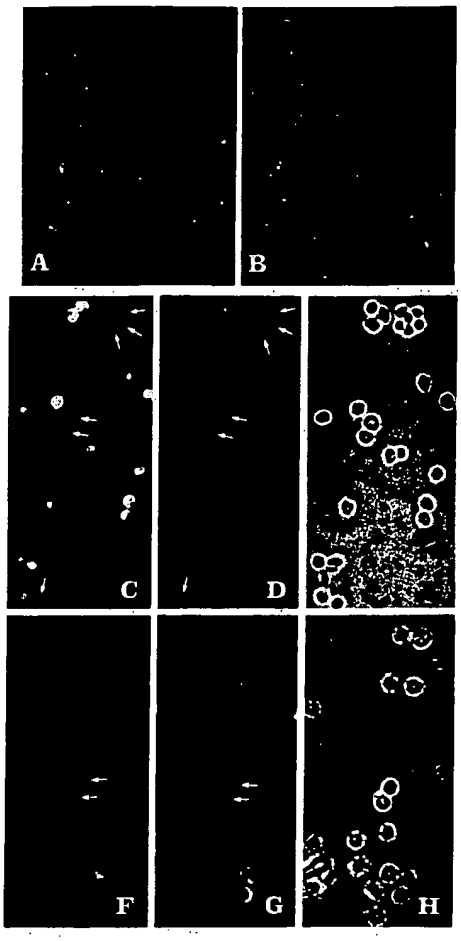

FIG. 1. Expression of I_i molecules by murine epidermal LCs. Epidermal sheets (*A* and *B*) were double-stained with mAb In1, anti-I_i (*A*), and mAb B21-2, anti-I-$A^{b,d}$ (*B*). Note that all class II-expressing cells also bear I_i and vice versa. Enriched populations of fresh (*C–E*) and 3-day-cultured LCs (*F–H*) attached to poly-L-lysine-coated slides were double-stained with mAbs In1 (*C* and *F*) and B21-2 (*D* and *G*). The expression of I_i chains decreases with culture and is almost absent in LCs cultured for 3 days (*C* vs. *F*), whereas class II expression increases (*D* vs. *G*). Note that all photographs were exposed and developed identically to allow for a semiquantitative comparison of fluorescence intensities. Arrows, keratinocytes. (*E* and *H*) Corresponding phase-contrast pictures. (×100.)

number of sialic acid residues bound per I_i molecule decreases. Cultured LCs as well as spleen DCs, a macrophage, and a B-cell line carry 9–11 acidic spots per I_i31 chain as opposed to up to 17 in freshly isolated LCs (Fig. 3 *B–E*). The molecular weight of the most sialylated I_i31 chains of cultured LCs remains below 40,000 and their negative charge resembles that of class II α chains. Class II molecules were always less sialylated (three or four acidic spots) than I_i chains (Fig. 3). It is emphasized that they, when opposed to actin as an internal reference standard, did not strongly vary in their charge (i.e., sialylation) between LCd0, LCd1, spleen DCs, macrophages, and B cells.

Comparison of Molecular and Functional Properties of Immature and Mature DCs. The exclusive functional property of mature DCs to sensitize resting T cells was frequently attributed to the nature of class II molecules on these cells (30, 31). However, biochemical analysis of surface radioiodinated LCs (data not shown) revealed no unusual pattern of surface class II molecules of mature DCs as compared to macrophages or B cells. Alternatively, fresh LCs were metabolically pulse labeled and chased for 12 or 32 hr to ensure

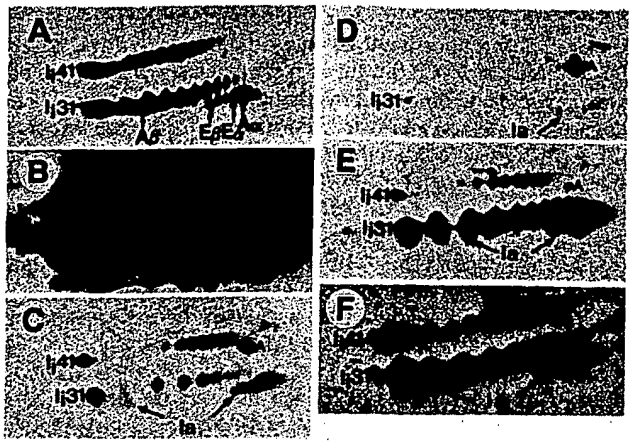

FIG. 2. Biosynthesis of class II and invariant chain polypeptides in LCs and DCs. LCd0 (*A*, *B*, and *F*), LCd1 (*C*), LCd3 (*D*), and spleen DCs (*E*) were metabolically labeled for 1 hr with [^{35}S]methionine and lysates were immunoprecipitated with anti-class II (*A–E*) and anti-I_i (*F*) mAbs. To demonstrate the decreasing intensities of the two-dimensional pattern, the gels in *B–E* were exposed to x-ray films for 25 days. In addition, the two-dimensional gels in *A* and *F* were exposed for 1 day. Class II chains are denoted with Aβ, Eβ, Eα, Aα (*A*) or with Ia (*C–F*). I_i41 and I_i31 are coprecipitated invariant chains. Note the marked decrease in biosynthesis upon culture of LCs (*B–D*). Assignment of the spots was done by using defined class II/I_i transfectants (22). A, actin.

expression of radiolabeled molecules on the cell surface (32). The same populations were also used to study antigen-presenting cell function. Anti-class II immunoprecipitates of 12-hr chased LCs (i.e., functionally immature LCs; Table 1) or 32-hr chased LCs (i.e., functionally mature LCs; Table 1) gave virtually identical two-dimensional gels (Fig. 4 *A* and *B*). There was also no striking qualitative difference to class II of peritoneal macrophages (Fig. 4).

Turnover of MHC Class II and I_i Molecules. This was studied in pulse/chase experiments of metabolically labeled cells. Class II molecules of LCs were more stable than those of macrophages: after a 12- or 32-hr chase period, the amounts of precipitable class II molecules were similar in LCs indicating that they had not decreased during this

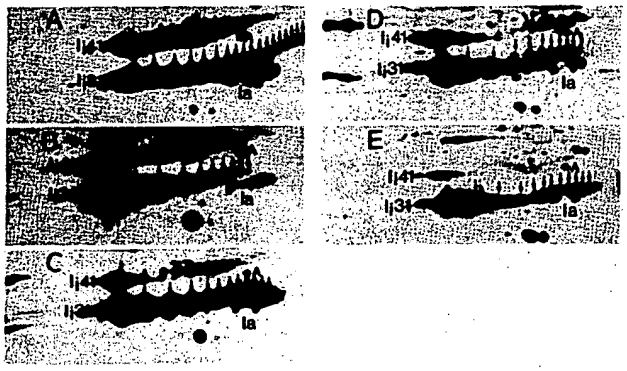

FIG. 3. Glycosylation patterns of I_i. Two-dimensional gels of I_i immunoprecipitates were exposed to x-ray films for various times to obtain similar intensities of invariant chains. (*A*) LCd0, 2 days. (*B*) LCd1, 25 days. (*C*) Spleen DC, 25 days. (*D*) Macrophage line P388D1 [preincubated with interferon γ (50 units/ml) to induce class II molecules], 20 days. (*E*) Pre-B-cell clone 18-81H6 (class II-positive variant of the 18-81 cell line), 10 days. The number of acidic spots of the I_i31 chain is marked by arrows. The ratio of I_i41/I_i31 is highest in fresh LCs (*A*) and lowest in B cells (*E*). Bands visible on the far left of *C* and *D* are ^{14}C molecular weight standards of M_r 27,000 and 46,000. Ia, class II molecules; A, actin.

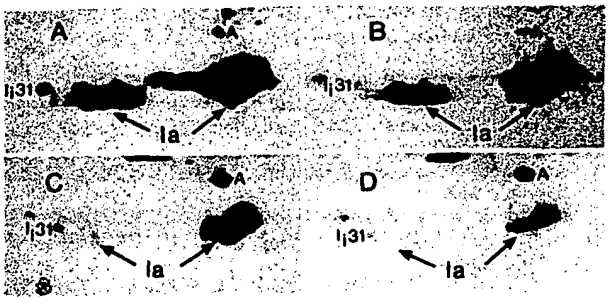

FIG. 4. Pulse/chase labeling of class II molecules in LCs and peritoneal macrophages. Freshly isolated LCs (*A* and *B*) and peritoneal macrophages (*C* and *D*) were pulse labeled for 15 min with [^{35}S]methionine and subsequently cultured for 12 hr (*A* and *C*) or 32 hr (*B* and *D*). Class II polypeptides (Ia and arrows) were immunoprecipitated with mAb 17/227. Note that only low amounts of I_i are present because of its rapid turnover and dissociation from class II. A, actin.

interval (Fig. 4 *A* and *B*). In contrast, markedly less class II was precipitated from macrophages after a 32-hr chase than after a 12-hr chase, suggesting a shorter half-life (Fig. 4 *C* and *D*). Densitometric scans of the gels in Fig. 4 demonstrated a 55% reduction of precipitable material (i.e., class II molecules) in macrophages over a period of 20 hr but no reduction in LCs. I_i molecules showed a different turnover. In freshly prepared LCs, they are synthesized in extremely large amounts as compared to other cell types (Fig. 2 *B–E*). The amount of I_i decreased sharply during the chase period, which is consistent with a short half-life of I_i (compare Fig. 5 *A* and *B* with Fig. 2*F*). It is remarkable that in LCs—as opposed to macrophages—I_i41 was relatively more stable than I_i31. Although initially more I_i31 than I_i41 was made by fresh LCs (Fig. 2*F*), most of the I_i31 had disappeared after a pulse/chase of 12 or 32 hr, whereas I_i41 remained precipitable with In1 (Fig. 5 *A* and *B*). In macrophages, neither chain was precipitable after the chase (Fig. 5 *C* and *D*).

DISCUSSION

DCs can efficiently activate resting T lymphocytes and are therefore key antigen presenting cells (1). It is unclear whether and how this capacity relates to the nature of MHC class II molecules. Also, the phenomenon of DC maturation/differentiation in culture (loss of antigen processing capacity and acquisition of stimulatory function) (3) needed to be studied in detail at the level of class II biochemistry. This

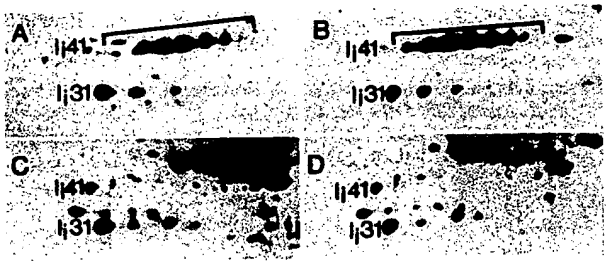

FIG. 5. Pulse/chase labeling of invariant chains in LCs and peritoneal macrophages. Cells were prepared and labeled as in Fig. 4. I_i chains were immunoprecipitated with In1. In both cultured LCs (*A* and *B*) and in macrophages (*C* and *D*) only marginal amounts of sialylated I_i31 chains and small amounts of unsialylated precursors are present. This indicates a high turnover rate of I_i31. In contrast, sialylated I_i41 in LCs is more abundant than I_i31, although synthesis of I_i31 in fresh LCs exceeds that of I_i41. Associated class II is hardly detectable. A spot to the left of I_i41 and I_i31 of macrophages is distinct from I_i chains and was not identified.

report confirms and extends recent work by Puré *et al.* (8) by using two-dimensional analyses and by focusing on structural features of the invariant chain.

Biochemistry of Class II and I_i Molecules of Immature DCs—Significance for Antigen Processing. LCs *in situ* or freshly isolated from the epidermis have some capacity to endocytose (8, 33, 34). They are equipped with acidic organelles like endosomes (10), the necessary cellular compartments for antigen processing (11, 12). The high rate of class II synthesis gives immature DCs the possibility to charge a large number of class II molecules with immunogenic peptides. The concomitant abundant synthesis of I_i, which is also involved in antigen processing (22, 26), would ensure that the peptide binding groove (35, 36) of all *de novo*-synthesized class II molecules is protected until the class II/I_i complex reaches the organelle where it encounters the antigen, presumably the acidic endosome (11, 12). The unusually high degree of I_i sialylation in fresh LCs gives these polypeptides a strong negative charge. This could promote trafficking/targeting and protease stability of the class II/I_i complexes, thereby facilitating their entry into the acidic processing organelles (27, 37). The significance of the high I_i41/I_i31 ratio is unknown at present. Taken together, these biochemical features may explain the extraordinary antigen-processing capacity of immature DCs as compared to other types of antigen presenting cells (7–9, 33).

Biochemistry of Class II and I_i Molecules of Mature DCs—Significance for T-Cell Sensitization. The cellular mechanism responsible for the unique capacity of mature DCs to sensitize resting T cells is presumably their ability to bind T cells in an antigen-*in*dependent fashion (38). It has not been possible so far to block this type of DC–T-cell interaction with antibodies to defined adhesion molecules (39–41). Moreover, the high density of class II molecules on mature DCs does not account for this binding property (5). Therefore, one may either postulate a hitherto unknown "clustering molecule" or, alternatively, obvious differences in class II molecules. Addressing the latter possibility, analysis of two-dimensional gels did not reveal such differences between class II molecules of antigen presenting cells that can (cultured LCs, spleen DCs) or cannot (fresh LCs, macrophages, B cells) bind T cells antigen independently. Low sialylation of surface class I and II has been discussed to be responsible for the immunostimulatory power of mature DCs (30, 31). However, we found no differences of class II sialylation patterns of immature and mature DCs and of B cells. Also, neuraminidase treatment of fresh LCs (F.K., N.R., and G.S., unpublished data) and macrophages (42) did not endow these cells with a T-cell sensitizing capacity. Thus, the putative molecule responsible for the initial antigen-independent binding of mature DCs to resting T cells remains unknown. Our data make it seem unlikely that it is merely a modification of class II.

Regulation of Class II and I_i Synthesis. Using flow cytometry it was shown that the increase in class II expression upon culture of LCs was not dependent on the cytokines granulocyte/macrophage colony-stimulating factor and tumor necrosis factor α (14). Also, interleukin 4 and/or interferon γ do not appear to be responsible cytokines because the up-regulation of class II expression occurs also in the absence of these factors (14). Cytokine effects on the synthesis of class II/I_i as measured by metabolic labeling of DCs have not yet been investigated.

LC Culture as a Physiologic Model for Antigen Presentation *in Vivo*. There is evidence that antigen-laden LCs can migrate from the skin to the draining lymph node (43, 44). It is tempting to speculate that before or during this migration LCs shut off class II/I_i synthesis, thereby preventing the possibility that by continuous processing the cells displace those immunogenic peptide/class II complexes that they have

formed while still in the epidermis. Indeed, both types of mature DC studied, cultured LCs and spleen DCs, have virtually stopped making class II and I_i. Their I_i molecules (Figs. 1 and 5) as well as their acidic organelles (10) rapidly decreased. In contrast, class II molecules were relatively stable in LCs as compared to murine B-cell lines and macrophages (45, 46). This very slow turnover of class II would make DCs well suited to carry antigenic peptides bound to class II to the lymphoid organs over a period of several days and efficiently present them to the T cells there. Indeed, it was recently shown that antigen-pulsed DCs can retain immunogenic peptide for at least 2 days *in vitro* (8) and *in vivo* (33).

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MOLECULAR BIOLOGY OF
THE CELL

SECOND EDITION

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into normal fibroblasts, the transfected cells are killed by the cytotoxic T cells described in the first experiment. These and other experiments suggest that fragments of viral proteins can both find their way to the cell surface and associate with class I MHC molecules.

It is not difficult to understand how viral proteins can be degraded in infected cells, since almost all cellular proteins are known to be continually degraded (see p. 418). It is more difficult to understand how fragments of the influenza nucleoprotein get to the cell surface, since the protein is synthesized on cytoplasmic ribosomes and would not normally have access to the lumen of the endoplasmic reticulum, where proteins destined for the cell surface usually begin their journey (see p. 412). T cell recognition probably requires very small amounts of antigen, however, so misrouting only a small fraction of the nucleoprotein fragments to the cell surface may create a target cell that an appropriate cytotoxic T lymphocyte can recognize.

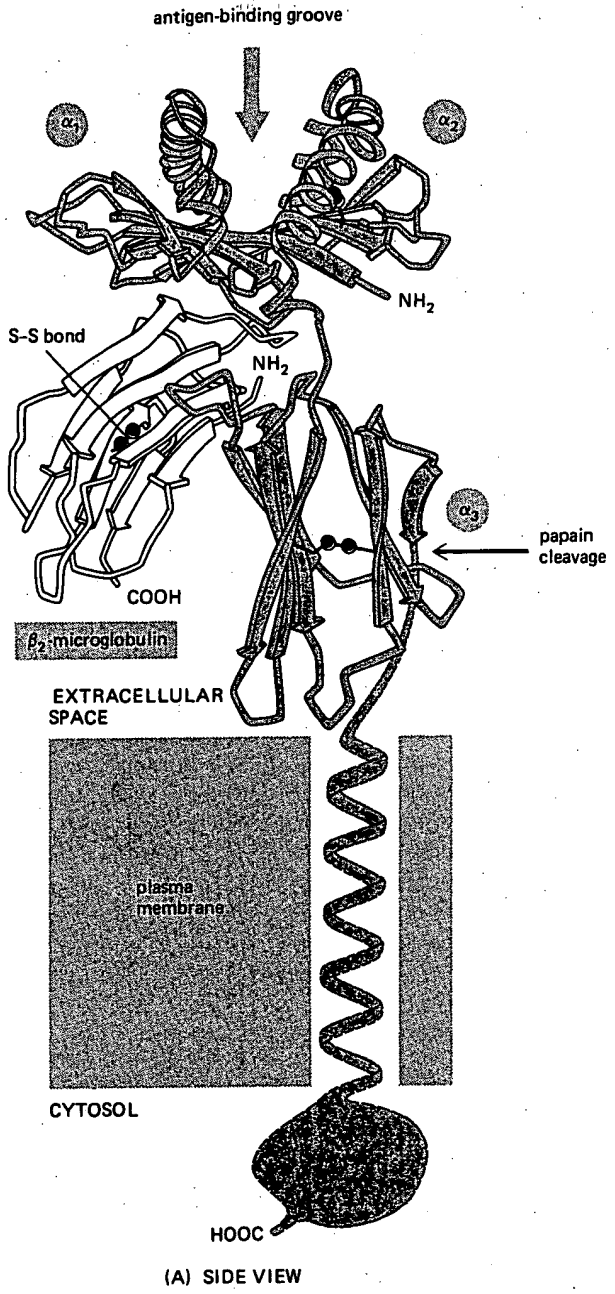
X-ray Diffraction Studies Show the Antigen-binding Site of a Class I MHC Glycoprotein[40]

A major advance in our understanding of how MHC molecules present antigen to T cells came in 1987, when the three-dimensional structure of a human class I MHC glycoprotein was obtained by x-ray crystallography. As shown in Figure 18–53A, the protein has a single putative antigen-binding site located at one end of the molecule. The site consists of a deep groove between two long α helices derived from the nearly identical α_1 and α_2 domains; the base of the groove is formed by eight β strands derived from the same two domains. The size of the groove is about 2.5 nm long, 10 nm wide, and 11 nm deep, which is large enough to accommodate a peptide of about 10 to 20 amino acid residues, depending on the extent to which the peptide is compressed by coiling or bending. Remarkably, the groove in the crystallized protein was not empty: it contained a small molecule of unknown origin, suspected to be a peptide, which co-purified and co-crystallized with the MHC glycoprotein (see Figure 18–53B). This finding strongly implicates the groove as the antigen-binding site and suggests that once a peptide binds to this site, it dissociates very slowly; this conclusion is supported by the observation that fibroblasts exposed for a short period to fragments of the influenza virus nucleoprotein remain targets for influenza-specific cytotoxic T cells for at least 3 days.

Most of the polymorphic amino acid residues in the MHC glycoprotein (those that vary between allelic forms of this type of molecule) are located inside the groove, where they would be expected to bind antigen, or on its edges, where they would be accessible for recognition by the T cell receptor. Presumably the variability in class I MHC molecules has been selected to allow them to bind and present many different virus-derived peptides. Nonetheless, it is still surprising that the small number of different antigen-binding sites associated with the class I MHC molecules in an individual (a maximum of six in humans) can bind the large number of virus-derived peptides that T cells can specifically recognize. Even more puzzling in this respect are the class II MHC glycoproteins, which are thought to have a three-dimensional structure very similar to that of class I molecules. Although an individual makes only about 10 to 20 types of class II molecules, each with its own unique antigen-binding site, these molecules seem to be able to bind and present an apparently unlimited variety of foreign peptides to *helper T cells*, which play a crucial part in almost all immune responses.

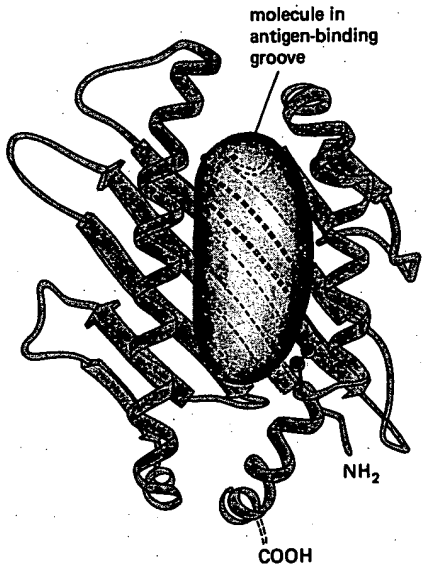
Helper T Cells Recognize Fragments of Foreign Antigens in Association with Class II MHC Glycoproteins on the Surface of Antigen-presenting Cells[41]

Helper T cells are required for most other types of lymphocytes to respond optimally to antigen. The crucial importance of helper T cells in immunity is dramatically demonstrated by the devastating epidemic of *acquired immunodeficiency syndrome (AIDS)*. The disease is caused by a retrovirus (human immuno-



(A) SIDE VIEW

Figure 18–53 (A) The structure of a human class I MHC glycoprotein as determined by x-ray diffraction analysis of crystals of the extracellular part of the molecule. The extracellular part was cleaved from the transmembrane segment by the proteolytic enzyme papain. Each of the two domains closest to the plasma membrane (α_3 and β_2-microglobulin) resembles a typical immunoglobulin domain (see Figure 18–28B), while the two domains farthest from the membrane (α_1 and α_2) are very similar to each other and together form a groove at the top of the molecule that is believed to be the antigen-binding site. Class II MHC molecules are thought to have a very similar structure. (B) The putative antigen-binding groove viewed from above, containing the small molecule (thought to be a peptide) that co-purified with the MHC protein. This is also the part of the molecule that interacts with the T cell receptor. (After P.J. Bjorkman, M.A. Saper, B. Samraoui, W.S. Bennett, J.L. Strominger, and D.C. Wiley, *Nature* 329:506–512, 1987.)



(B) TOP VIEW

deficiency virus, HIV) that kills helper T cells, thereby crippling the immune system and rendering the patient susceptible to infection by microorganisms that rarely infect normal individuals. As a result, most AIDS patients die of infection within several years of the onset of symptoms.

Before they can help other lymphocytes respond to antigen, helper T cells must first be activated themselves. This activation occurs when a helper T cell recognizes a foreign antigen bound to a class II MHC glycoprotein on the surface of a specialized **antigen-presenting cell.** Antigen-presenting cells are found in most tissues. They are derived from bone marrow and comprise a heterogeneous set of cells, including *dendritic cells* in lymphoid organs, *Langerhans cells* in skin, and certain types of macrophages. Together with B cells, which can also present antigen to helper T cells (see below), and thymus epithelial cells (see p. 1052), these specialized antigen-presenting cells are the main cell types that normally express class II MHC molecules (see Table 18–2).

Many kinds of experiments demonstrate the central importance of class II MHC molecules in presenting foreign antigens to helper T cells. The binding of antibodies to class II molecules on antigen-presenting cells, for example, blocks

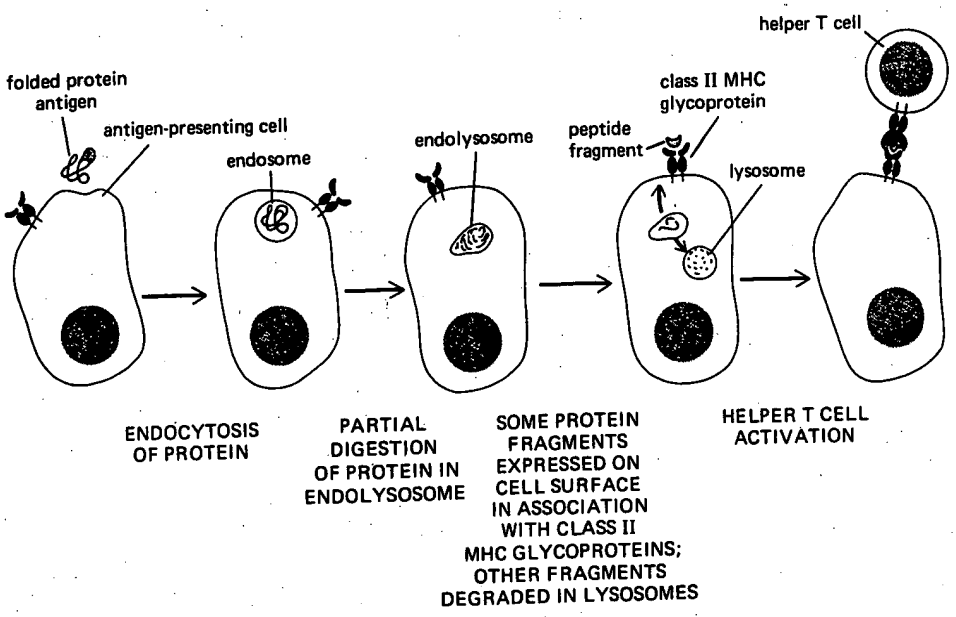


Figure 18–54 How protein antigens are thought to be "processed" and then displayed by an antigen-presenting cell. Since class II MHC glycoproteins have been found to recycle through the endosomal compartment, they may initially associate with peptide fragments in the endolysosomal compartment and then return to the cell surface with a bound peptide (not shown).

the ability of these cells to present foreign antigen to helper T cells. Moreover, fibroblasts, which do not make class II MHC molecules and cannot present foreign antigens to helper T cells, can be converted to effective antigen-presenting cells if they are transfected with a gene that codes for a class II MHC molecule.

Like the viral antigens presented to cytotoxic T cells, the antigens presented to helper T cells on antigen-presenting cells are usually degraded fragments of the foreign protein. These peptides are thought to be bound to class II MHC molecules in the same way that virus-derived peptides are bound to class I MHC molecules (see Figure 18–53). Unlike the virus-infected target of a cytotoxic T cell, however, the antigen-presenting cell does not synthesize the foreign protein. Instead, it is thought that the foreign protein is ingested by endocytosis and partially degraded in the acidic environment of endosomes or endolysosomes (see p. 331) before selected fragments are returned to the cell surface—a sequence of events collectively called **antigen processing** (Figure 18–54). Thus, if endocytosis is blocked by lightly fixing antigen-presenting cells with a chemical such as formaldehyde, or if proteolysis in endolysosomes and lysosomes is inhibited by a drug such as chloroquine, the cells are no longer able to process a foreign protein and present it to helper T cells. Cells treated in these ways, however, are still able to present the protein if it is cleaved into small peptides (10–15 amino acids long) before it is added to the cells.

A remarkable property of an antigen-presenting cell is that it can process and present virtually any antigen to an appropriate helper T cell. This lack of antigen specificity suggests that antigen-presenting cells take up antigen by fluid-phase rather than by receptor-mediated endocytosis (see p. 328). If this is so, then most of the proteins ingested and degraded will be host (self) proteins, whose peptide fragments will occupy the binding site of many of the class II MHC molecules. Presumably the binding of foreign peptides to only a small proportion of MHC molecules is sufficient to activate a helper T cell.

Helper T Cells Stimulate Activated T Lymphocytes to Proliferate by Secreting Interleukin-2[42]

Activation of a helper T cell is a complex process involving various secreted proteins called **interleukins,** which act as local chemical mediators. Activation is thought to begin when the T cell, by unknown means, stimulates the antigen-presenting cell to secrete one or more interleukins. The best characterized of these mediators is **interleukin-1 (IL-1).** The combined action of IL-1 (and probably other interleukins) and antigen binding, however, do not stimulate helper T cell proliferation directly. Instead, they cause the T cell to stimulate its own proliferation by inducing it to secrete a growth factor called **interleukin-2 (IL-2)** as well as to

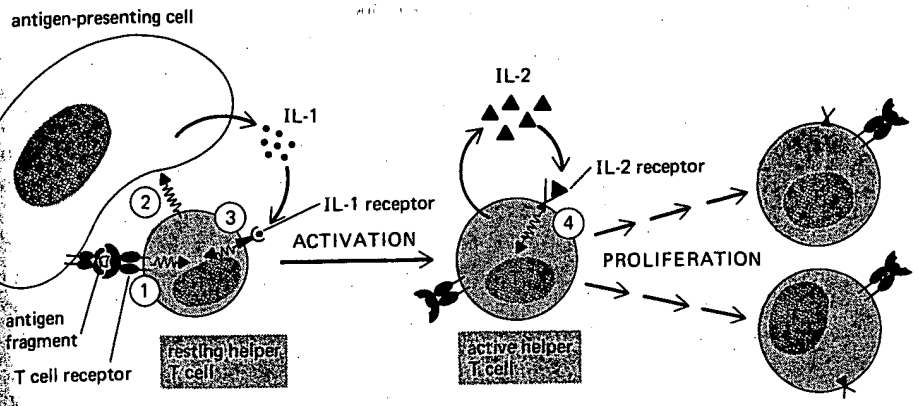


Figure 18–55 The sequence of signaling events believed to occur when antigen stimulates helper T cells to proliferate. Binding of the T cell to the antigen on the surface of an antigen-presenting cell signals the T cell receptor to trigger the inositol phospholipid cell-signaling pathway (see p. 702) (signal 1). This causes the T cell to stimulate the antigen-presenting cell by an unknown mechanism (signal 2). The antigen-presenting cell then secretes interleukins, such as interleukin-1 (IL-1), which help activate the T cell (signal 3). The activated T cell makes interleukin-2 (IL-2) receptors and secretes IL-2; the binding of IL-2 to its receptors (signal 4) stimulates the cell to grow and divide. When the antigen is eliminated, the T cells eventually stop producing IL-2 and IL-2 receptors, so cell proliferation stops.

synthesize cell-surface IL-2 receptors. It is the binding of IL-2 to these receptors that stimulates the T cell to proliferate. In this way the helper T cell can continue to proliferate, through an *autocrine mechanism* (see p. 690), after it has left the surface of the antigen-presenting cell (Figure 18–55). The helper T cell can also help stimulate the proliferation of any other T cells, including cytotoxic T cells, that have first been induced to express IL-2 receptors. Because the expression of IL-2 receptors is strictly dependent on antigen stimulation, however, this does not result in the indiscriminate proliferation of all T cells, but only those that have encountered antigen.

Once the requirements for T cell proliferation were discovered, it was possible to produce indefinitely proliferating, antigen-specific *T cell lines* in culture by continuously administering IL-2 and periodically stimulating the cells with antigen to maintain the expression of IL-2 receptors. Single cells from such lines could then be isolated to generate **T cell clones.** As we have seen, such clones have been critically important in T cell research. They made it possible, for example, to isolate T cell receptors and their genes; they have also been widely used to study the mechanisms of T cell activation and the role of helper T cells in stimulating the responses of other lymphocytes.

Helper T Cells Are Required for Most B Cells to Respond to Antigen[43]

Helper T cells are essential for B cell antibody responses to most antigens. This was first discovered in the mid-1960s through experiments in which either thymus cells or bone marrow cells were injected together with antigen into irradiated mice. Mice that had received only bone marrow or only thymus cells were unable to make antibody; but if a mixture of thymus and bone marrow cells was injected, large amounts of antibody were produced. It was later shown that the thymus provides T cells, while the bone marrow provides B cells (Figure 18–56). The use of a specific chromosome marker to distinguish between the injected T and B cells showed that the antibody-secreting cells are B cells, leading to the conclusion that T cells must help B cells respond to antigen.

There are some antigens, however, including many microbial polysaccharides, that can stimulate B lymphocytes to proliferate and mature without T cell help. Such *T-cell-independent antigens* are usually large polymers with repeating, identical antigenic determinants whose multipoint binding to the membrane-bound antibody molecules that serve as antigen receptors on B cells may generate a strong enough signal to activate B cells directly. There is evidence that the cells that respond to multimeric antigens in this way are mainly a separate subset of B cells that has evolved to react against microbial polysaccharides without T-cell help.

Helper T Cells Help Activate B Cells by Secreting Interleukins[44]

Once activated by foreign antigen on the surface of a specialized antigen-presenting cell, an appropriate helper T cell can help activate a B cell by binding to the same foreign antigen on the B cell surface. The antigen-presenting cell ingests and presents antigens nonspecifically (see p. 1046), but a B cell generally

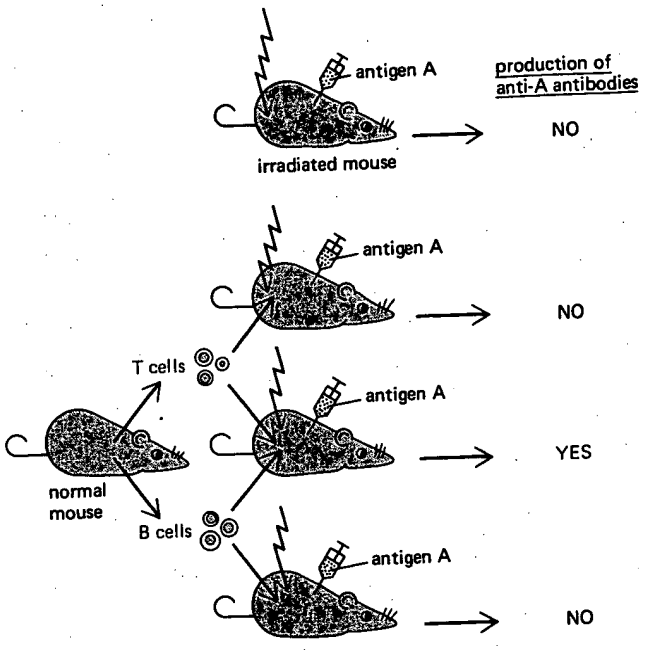


Figure 18–56 The experiment that first suggested that both T cells and B cells are required if an animal is to make antibody responses. The dose of irradiation used kills the T cells and B cells of the irradiated mouse.

presents only an antigen that it specifically recognizes. The antigen is selected by its binding to the specific membrane-bound antibodies (antigen receptors) on the surface of the B cell; it is ingested by receptor-mediated endocytosis (see p. 328) and is then degraded and recycled to the cell surface in the form of peptides bound to class II MHC glycoproteins for recognition by the helper T cell. Thus the helper T cell recognizes the same antigen-MHC complexes on the B cell it helps as on the antigen-presenting cell that initially activated the T cell.

The specific contact between a helper T cell and a B cell initiates an internal rearrangement of the helper cell cytoplasm that orients the centrosome and Golgi apparatus toward the B cell, as described previously for a cytotoxic T cell contacting a target cell (see Figure 18–47). In this case, however, the orientation is thought to enable the helper T cell to direct the secretion of interleukins (and perhaps to focus membrane-bound signaling molecules) onto the B cell surface. These interleukins include *IL-4*, which helps initiate B cell activation, *IL-5*, which stimulates activated B cells to proliferate, and *IL-6*, which induces activated B cells to mature into antibody-secreting cells. Some of these and other interleukins can induce B cells to switch from making one class of antibody to making another (see p. 1029). Some of the signals thought to be involved in the initial activation of a B cell are illustrated in Figure 18–57.

How do signals pass from activated cell-surface receptors to the cell interior when B or T cells are stimulated by antigen and interleukins? The answer is not known for interleukin receptors, but there is strong evidence that receptors for antigen on both B and T cells signal the cell by activating the inositol phospholipid pathway discussed in Chapter 12 (see p. 702).

Some Helper T Cells Activate Macrophages by Secreting γ-Interferon[45]

Helper T cells do not confine their help to lymphocytes. Those helper T cells that secrete IL-2 when stimulated by antigen also secrete other interleukins, such as *γ-interferon*, that attract macrophages and activate them to become more efficient at phagocytosing and destroying invading microorganisms. The ability of T cells to attract and activate macrophages is especially important in defense against infections by microorganisms that can survive simple phagocytosis by nonactivated macrophages. Tuberculosis is one such infection.

The antigen-triggered secretion of γ-interferon and other macrophage-activating interleukins by helper T cells underlies the familiar tuberculin skin test. If tuberculin (an extract of the bacterium responsible for tuberculosis) is injected

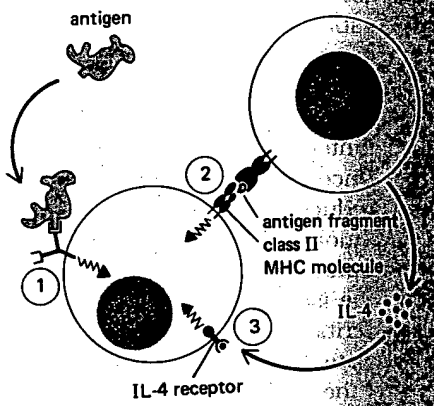


Figure 18–57 At least three types of signals are likely to be involved in the initial stages of B cell activation. The relative importance of these signals is uncertain and may vary depending on the type of B cell and antigen. Signal 1 is caused by antigen binding and is thought to be mediated by the inositol phospholipid cell-signaling pathway (see p. 702); it helps activate the B cell and may induce the expression of receptors for some of the helper-T-cell-derived interleukins. The B cell then ingests and degrades the antigen (not shown) and presents small fragments of the antigen to the helper T cell in association with class II MHC molecules. It is not clear if T cell binding signals the B cell (shown here as signal 2) or only serves to focus the secretion of interleukin-4 (IL-4) and other interleukins (not shown) onto the B cell surface (signal 3). In addition to activating the B cell, signal 3 stimulates the cell to make more class II MHC glycoprotein, thereby increasing the ability of the B cell to receive T cell help. Once the B cell is activated, other helper-T-cell-derived interleukins (such as IL-5, IL-6, and γ-interferon) help induce the cell to proliferate and mature into an antibody-secreting cell (not shown).

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Immunology

Heteroclitic proliferative responses and changes in cytokine profile induced by altered peptides: Implications for autoimmunity

(costimulation/superagonists/T cell differentiation)

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ABSTRACT **Productive engagement of T cell receptors (TCRs) by cognate ligand (major histocompatibility complex plus peptide) leads to proliferation, differentiation, and the elaboration of effector functions. Altered peptides generated by single amino acid substitutions in the antigenic peptide have diverse effects on the outcome of the T cell response. We have generated an altered peptide (Q144) from an autoantigenic peptide of myelin proteolipid protein 139–151 by a single amino acid substitution (from tryptophan to glutamine) in the primary TCR contact at position 144 that is capable of inducing CD4+ T cell responses in H-2^s mice. By using a Q144-specific T cell clone (Q1.1B6), we see a hierarchy in T cell proliferation and cytokine production with various position 144 substituted peptides and have identified a peptide (L144) that hyperstimulates this T cell clone. In contrast to Q144, L144 induces maximal proliferation at 7 logs lower antigen concentration, induces greater cell death at higher antigen dose, and induces the secretion of cytokines not detected following stimulation with the cognate ligand. This heteroclitic T cell response associated with changes in cytokine profile was observed with several other T cell clones of different specificities. The L144 peptide also induces costimulation independent proliferation and cytokine production from the Q1.1B6 T cell clone. We describe this as a superagonist response. Such responses may have a role in the initiation of autoimmunity by promoting a proinflammatory environment following ligation of a cross-reactive TCR on autoreactive T cells.**

The effector functions of T cells are dictated, to a large extent, by the cytokines produced by the cell following activation and differentiation (1). Cytokines can initiate, propagate, or regulate tissue-specific autoimmune injury. In a number of autoimmune disease models, including experimental autoimmune encephalomyelitis (EAE), T helper type 1 (Th1) cells that secrete pro-inflammatory cytokines [interleukin 2 (IL-2), interferon γ (IFN-γ), and tumor necrosis factor β (TNF-β)] induce autoimmunity (2), whereas Th2 cells (secreting IL-4 and IL-10) can protect from EAE, although they do not always do so (3–6). The phenotype of the CD4+ T cell response in human disease has also been correlated with the outcome of infection and the autoimmune process (7). Much of the experimental work in models of autoimmunity has focused on the immune response to specific peptide ligands (cognate ligands), but it is also known that subtle modification of these antigens [to produce altered peptide ligands (APLs)] can have profound effects on the outcome of disease (8–11).

How APLs alter the course of autoimmune disease has been an area of intense interest in recent years, and the *in vitro* effects of some altered peptides may begin to explain their *in vivo* functions. APLs have been shown to mediate T cell receptor (TCR) antagonism (12), induce T cell anergy (13), and partially activate T cell clones (14, 15). Some of our recent work (16, 17) and that of others (18) has suggested that APLs can affect T cell differentiation and therefore the Th1/Th2 balance may determine disease outcome. In an EAE model induced with proteolipid protein (PLP) peptide 139–151 (W144), we have identified peptide analogs that protect animals from disease (11, 16). For at least one analog in which the tryptophan at position 144 has been replaced with glutamine (Q144), the ability to transfer protection appears to be a function of a subset of T cells that are cross-reactive and respond to both Q144 and the native PLP peptide W144 (16). Therefore the cross-reactive nature of these responses seems to be critical to their effects *in vivo*.

Because of the biological significance of cross-reactive T cells in our experimental system, we characterized the response of cross-reactive T cell clones derived by immunization with Q144. We found a hierarchy in the response of clones specific for various altered ligands. Heteroclitic proliferative responses were associated with changes in the cytokine profile of the responding clones, and at equivalent peptide concentrations induced a more pro-inflammatory environment than the cognate ligand. We have characterized the response of one clone, Q1.1B6, in detail and have shown that the heteroclitic ligand demonstrates a hierarchy in the induction of secretion of different cytokines. Furthermore, activation with the "superagonist" ligand was less costimulation dependent than activation with the cognate ligand. The existence of similar superagonist ligands in nature may be important in the induction and/or regulation of autoimmune disease.

MATERIALS AND METHODS

Generation of T Cell Clones and Peptide Antigens. T cell clones were derived from female SJL mice (H-2^s) immunized with 100 μg of peptide antigen Q144 in complete Freund's

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Abbreviations: APC, antigen presenting cell; APL, altered peptide ligand; EAE, experimental autoimmune encephalomyelitis; IFN, interferon; MHC, major histocompatibility complex; TCR, T cell receptor; Th, T helper; IL, interleukin; TNF, tumor necrosis factor; ECDI, [1-ethyl-3-(3′-dimethylaminopropyl) carbodiimide].

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AF037038 and AF037039).

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adjuvant as described (16). Q1.1B6 was cloned twice and maintained in culture by stimulation with irradiated syngeneic spleen cells and Q144 (15 μM) every 1–2 months. Other clones were derived as described (17, 19). Peptide antigens with C terminal amides were synthesized by Richard Laursen (Boston University) on a MilliGen model 9050 synthesizer by using fluorenylmethoxycarbonyl chemistry. The peptides described have the sequences HSLGKWLGHPDKF (proteolipid protein 139–151/W144), HSLGKLLGHPDKF (L144), HSLGKRLGHPDKF (R144), HSLGKQLGHPDKF (Q144), HSLGKALGHPDKF (A144), and HSLGKLLGRPDKF (L144/R147). Substitutions are shown in bold.

Proliferation and Cytokine Assays. Rested T cell clones ($1–2 \times 10^4$ cells per well) were activated with irradiated (3,000 rads) syngeneic splenic antigen-presenting cells (APCs; 5×10^5 per well) and peptide antigens or anti-TCR or anti-CD3 (PharMingen) antibody. Proliferation was assessed by pulsing the cells with [^{3}H-methyl]thymidine 1 μC/well (1 Ci = 37 GBq) 48 h after activation. The cells were harvested 18 h later, and the incorporated radioactivity was measured in triplicate wells. Supernatants were collected 40 h after activation and diluted 1:2; then cytokine concentrations were measured by specific capture ELISA according to the manufacturers instructions (PharMingen) as described (16). To assess the response to fixed APCs and different peptides, syngeneic splenic APCs were irradiated and an aliquot of cells was fixed with 75 mM ECDI (CalBiochem-Novabiochem) for 1 h as described (20). The fixed and unfixed APCs were washed extensively and used with Q144 or L144 at 60 μM to activate Q1.1B6. Blockade of the CD28/CTLA4 pathway was performed with human CTLA4-Ig (Genetics Institute, Cambridge, MA) compared with activation in the presence of a control fusion protein.

TCR cDNA Cloning. cDNAs encoding TCR-α and -β chains were isolated by inverse PCR essentially as described (21). In brief, poly(A)$^+$ mRNA was isolated from 10^6 cells, primed with oligo(dT) 12–18 and transcribed to first-strand cDNA with SuperScript II RT (GIBCO/BRL). Double-stranded cDNA was synthesized by using a cDNA synthesis system (GIBCO/BRL) and subsequently blunt ended with T4 DNA polymerase (New England Biolabs). The cDNA was circularized by T4 DNA ligase (New England Biolabs) and subjected to PCR (94°C for 1 min, 55–57°C for 1 min, and 72°C for 2 min for 30 cycles) by using two C$^\alpha$- (AAGAGACCAACGCCACCTAC, GCTGTCCTGAGACCGAGGAT) or C$^\beta$-(GCACAATCCTCGAAACCACT, GATGGCTCAAACAAGGAGAC) specific primers. PCR products were ligated into plasmid pCR2.1 Vector (Invitrogen) and the recombinant DNA was electroporated into competent *Escherichia coli* XL1-Blue MRF′ (Stratagene). Positive transformants were identified by colony hybridization by using ^{32}P-labeled C$^\alpha$- or C$^\beta$-specific internal oligonucleotides, respectively, as probes. Their DNA was subsequently isolated and sequenced.

Measurement of Peptide Binding to I-A^s. I-A^s molecules were prepared by affinity chromatography from cell lysates derived from the B cell lymphoma LS102.9 (H-2^{dxs}), and the binding of various peptides was measured in a competition assay with a radiolabeled peptide as described (22, 23). The concentration of peptide needed to inhibit binding by 50% was calculated from this assay.

RESULTS

The Q1.1B6 clone was generated from SJL mice immunized with Q144 and responds to this peptide in the context of I-A^s. To probe the fine specificity of the response of this clone we activated it with a number of different position 144 substituted peptides, all of which have a similar affinity for I-A^s, and measured the proliferative response (Fig. 1). With these peptides we could define a hierarchy of responses. Two analogs

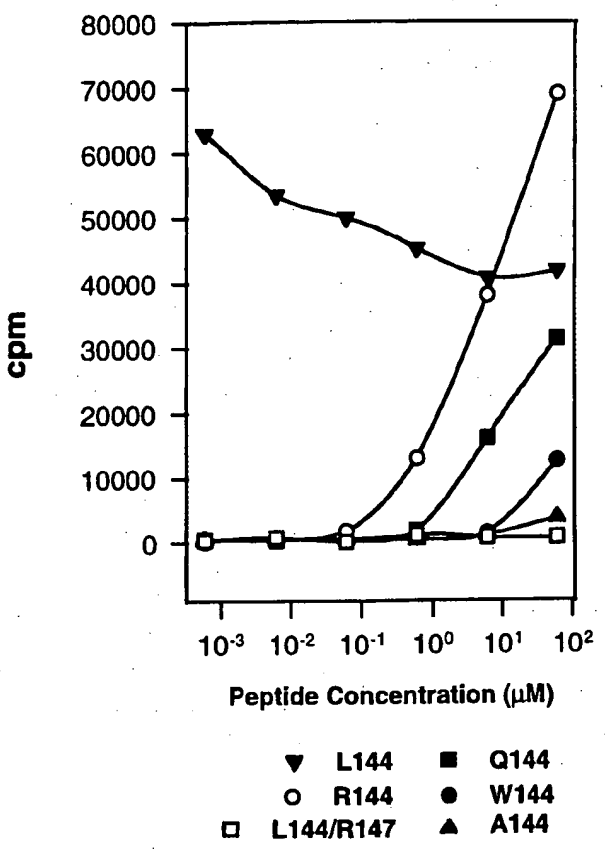

FIG. 1. Hierarchy of the T cell proliferative response of the T cell clone Q1.1B6 to different altered peptide ligands: L144 > R144 > Q144 >W144 ≥ A144 ≫ L144/R147. T cell clones were activated with peptide antigens at the concentrations shown. Proliferation was assessed 48 h after activation.

(L144, R144) elicited proliferation at lower concentrations than the cognate ligand (Q144), whereas higher concentrations of two others (W144, A144) were needed to induce a response. No proliferation was detected with the double substituted analog L144/R147. These APLs could be ranked relative to each other in terms of potency in the proliferation assay and the complete hierarchy of response was found to be L144 > R144 > Q144 > A144 ≥ W144. The response to L144 was particularly striking, because even at 6×10^{-4} μM the proliferation induced by the peptide had not reached a maximum and at higher peptide concentrations the peptide appeared to inhibit T cell growth. This heteroclitic behavior was noted with L144 synthesized at two different facilities and was T cell specific because the same L144 was nonantigenic with other independently derived Q144 specific T cell clones (data not shown).

To characterize the functional response further we measured cytokines in the supernatants of cells activated with various ligands (Table 1). The phenotype of the Q1.1B6 clone was Th0, because activation with Q144 stimulated the production of both IFN-γ and IL-4. Ligands lower in the hierarchy (A144, W144) induced the same cytokines as Q144 but in lower amounts, and W144 induced relatively more IFN-γ than IL-4. Surprisingly, the analogs that hyperstimulated the clones also induced the secretion of detectable levels of IL-2. L144 consistently stimulated IL-2 production, and activation with R144 also elicited lower levels of this cytokine. To confirm that the differences in detectable IL-2 reflected differences in its induction we assessed the amount of IL-2 mRNA following stimulation by 50 μM Q144 or L144 with quantitative reverse

Table 1. The pattern and amounts of cytokines produced by the Q1.1B6 clone changes following stimulation with different peptide analogs despite similar binding affinities for MHC class II

APL	Sequence	Relative MHC binding	Proliferation, Δcpm	Δ cytokine, pg/ml IFN-γ	IL-2	IL-4
Q144	HSLGKQLGHPDKF	0.6	84,250	**2,650**	<50	**1,090**
A144	HSLGKALGHPDKF	0.6	22,200	**730**	<50	**370**
L144	HSLGKLLGHPDKF	0.8	29,420	**13,770**	**1,490**	**9,420**
R144	HSLGKRLGHPDKF	0.8	48,650	**8,890**	**490**	**2,550**
W144	HSLGKWLGHPDKF	1.0	16,103	**2,160**	<50	**100**
L144/R147	HSLGKLLGRPDKF	1.3	37	<100	<50	<25

The data are the mean values from four or five independent experiments at antigen concentrations tested between 6 and 60 μM. Values >3× background are shown in bold.

transcription–PCR by using a cytokine mimic (24). This demonstrated a 100-fold increase in IL-2 mRNA levels after activation with L144 compared with Q144 (data not shown). These results were confirmed by intracytoplasmic staining of IL-2 (data not shown). IL-10 was detected in some experiments following activation with L144 and R144 but was never detected following activation by the cognate ligand or the other analogs (data not shown). This hyperstimulation is characterized by a heteroclitic proliferative response and the secretion of cytokines not detected following activation by the cognate ligand. We describe such hyperstimulatory ligands as superagonists.

To exclude the possibility that the response we observed was caused by the expression of multiple TCRs, we identified the TCR genes expressed by Q1.1B6 by inverse PCR. In-frame rearrangements corresponding to AV1S1Ja10 and BV10S1A2-Db1, Jb2.1 were identified for TCR-α and -β, respectively (Fig. 2). An additional out-of-frame rearrangement was identified for TCR-α. No other TCR-α rearrangements were detected among 32 PCR clones examined. Expression of TCR-β chains in Q1.1B6 was examined further by PCR by using primers specific for all reported Vβ sequences (25). This confirmed the presence of Vβ10, and no additional rearrangements were detected (data not shown). This shows that the clone expressed only one functional TCR-α and -β chain gene.

To test whether the effects of the superagonist peptide could be mimicked by increasing concentrations of the cognate ligand, we compared the response of Q1.1B6 to L144 over a broad dose range from 6×10^{-10} to 6×10^{1} μM peptide, with the response to Q144, in terms of proliferation and cytokine production. These experiments revealed a hierarchy in the induction of different cytokines with their half-maximal production occurring at very different concentrations of L144 (Fig. 3*A*). The most sensitive measure of T cell response was proliferation that was detectable a 6×10^{-10} μM L144 compared with 6×10^{-1} μM Q144. IFN-γ and IL-4 were the first cytokines detected. Their production reached a maximum within 1–2 logs of the concentration which elicited maximum proliferation. In the case of L144, further increases in peptide to 6×10^{-5} μM led to detectable IL-2 secretion and at the highest doses of L144 TNF-α could also be detected. The T cells were confirmed to be the source of TNF-α by demonstrating its detection from T cells stimulated by fixed APCs (see below). In contrast, in the same experiment the response to Q144 fell within a narrow concentration range (6×10^{-1} to 6×10^{2} μM) and only IFN-γ and IL-4 were detected. Neither at maximal proliferation nor at the highest antigen concentration tested (6×10^{2} μM of Q144) were IL-2 or TNF-α detected. Although the difference in reactivity to Q144 and L144 may be quantitative and not qualitative, to achieve with Q144 the stimulation we see with L144, we estimate that theoretically it would be necessary to dissolve 1 g of Q144 in 1 ml of medium to elicit IL-2 and 1 kg of peptide in 1 ml of medium to elicit TNF-α.

We then determined whether activation of Q1.1B6 with anti-TCR or anti-CD3 antibody plus APCs, with the potential to cross-link all the available TCRs, resembled more closely the response to Q144 or L144. With either antibody the proliferation reached a maximum comparable to that induced by Q144 and we detected IFN-γ and IL-4 but not TNF-α or IL-2. The data for anti-TCR-antibody is shown in comparison with Q144 peptide (Fig. 3*B*). We conclude that over the concentration range used, anti-TCR or anti-CD3 antibody was unable to elicit superagonist responses.

Because the normal activation of T cell clones requires cognate ligand (signal 1) and costimulation in the form of B7 (CD80/CD86)-CD28 mediated signaling (signal 2) (26, 27), we wished to determine whether superagonists had the same requirement. Activation with cognate ligand in the absence of signal 2 leads to T cell anergy (28), a process thought to limit activation of T cells by self antigens (29). If superagonists have different requirements for costimulation, they may have the potential to overcome this checkpoint in self-tolerance. To assess this we activated rested T cell clones with ECDI-fixed and non-fixed APCs. We found that on activation with fixed APCs, Q144 was only able to elicit low levels of IFN-γ secretion and did not induce significant proliferation or IL-4 production. Under the same fixation conditions L144 induced proliferation and production of all the cytokines detectable on stimulation with unfixed APCs (Fig. 4*A*). The average levels of proliferation were actually higher following activation by L144 on fixed APCs because of a reduction in the high-dose inhibition of growth seen with this peptide (Fig. 4*A*). To determine the specific role of the costimulation via CD28/CTLA4 we blocked activation with human CTLA4-Ig. At high concentrations of antigen this blockade had little effect fol-

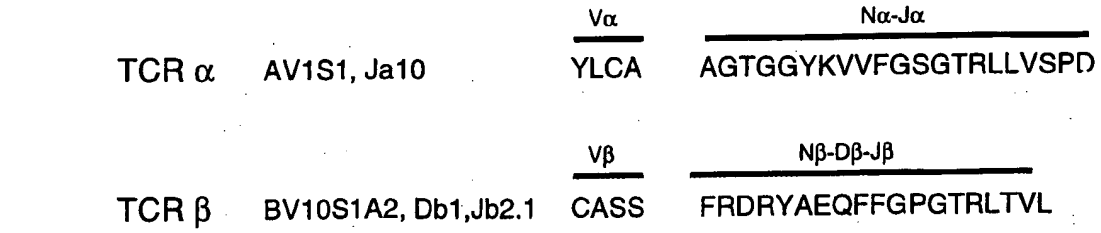

FIG. 2. TCR utilization by the Q1.1B6 clone. TCR V, D, and J segments and the predicted amino acid sequence of V(D)J regions of TCR-α and -β chains from T cell clone Q1.1B6 are shown. Assignments to V, D, and J segments are based on Arden *et al.* (40).

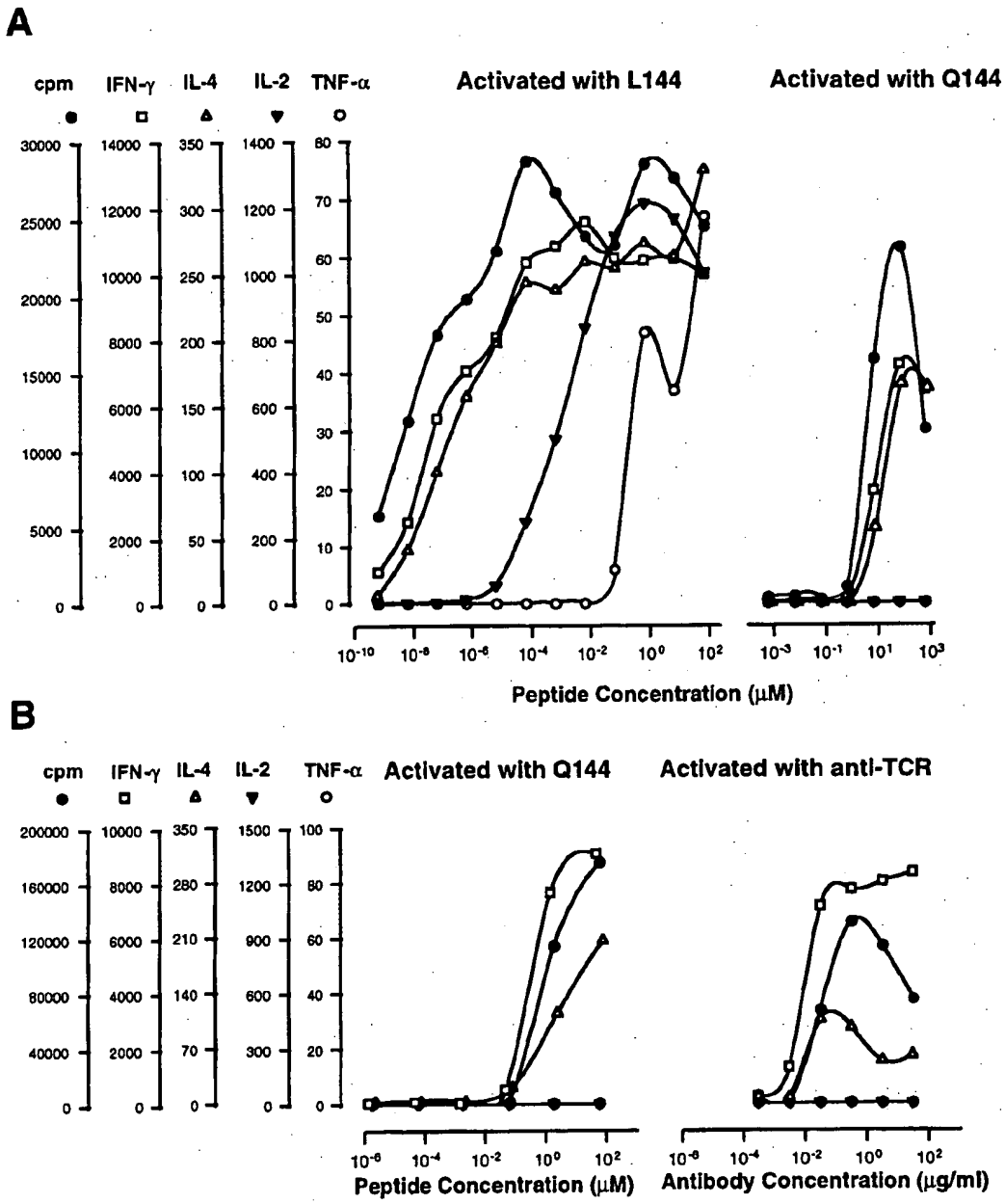

FIG. 3. Dose response of Q1.1B6 with agonist peptide (Q144), superagonist peptide (L144), and anti-TCR antibodies (H57.597). (*A*) L144 and Q144 differ in proliferation and pattern of cytokines produced from the Q1.1B6 clone over a wide dose range, following activation by antigen and APCs. (*B*) The proliferation and cytokine production to anti-TCR antibody most closely resembles the response to the cognate Q144 ligand. All samples in each set were tested on the same plate. Background proliferation was <400 cpm. Background cytokine production was below the limit of detection of the assays. One representative experiment of at least four is shown.

lowing activation with either Q144 or L144. At lower antigen concentrations activation by Q144, but not by L144 was significantly reduced by the CTLA4-Ig but not control fusion protein (Fig. 4*B*). These experiments have been confirmed by using CHO cells transfected with I-A^s alone, or I-A^s with B7 costimulatory molecules. Activation with Q144 requires costimulation whereas activation with L144 does not (A. Murtaza and V.K.K., unpublished data). We conclude that stimulation with the superagonist ligand L144 has less stringent costimulatory requirements than activation with the cognate ligand Q144. In fact, in some cases the presentation of L144 by costimulation-deficient APCs may actually induce greater expansion of Q1.1B6 compared with presentation on costimulation competent APCs.

To address the issue of whether heteroclitic T cell responses with changes in cytokine profile were only seen in one clone, Q1.1B6, or whether other clones would show similar hierarchies and changes in cytokine patterns, we analyzed T cell clones generated independently from mice immunized with altered peptide ligands (L144/R147 and Q144). We identified four clones from two additional clonings that produced significant amounts of additional cytokines (IL-2 and IFN-γ) on activation with the hyperstimulatory altered ligand, but not the immunizing ligand (Table 2). This demonstrates that several different clones from different clonings and of different specificities, shifted their cytokine profile toward the production of Th1 cytokines on activation with heteroclitic ligands. It also

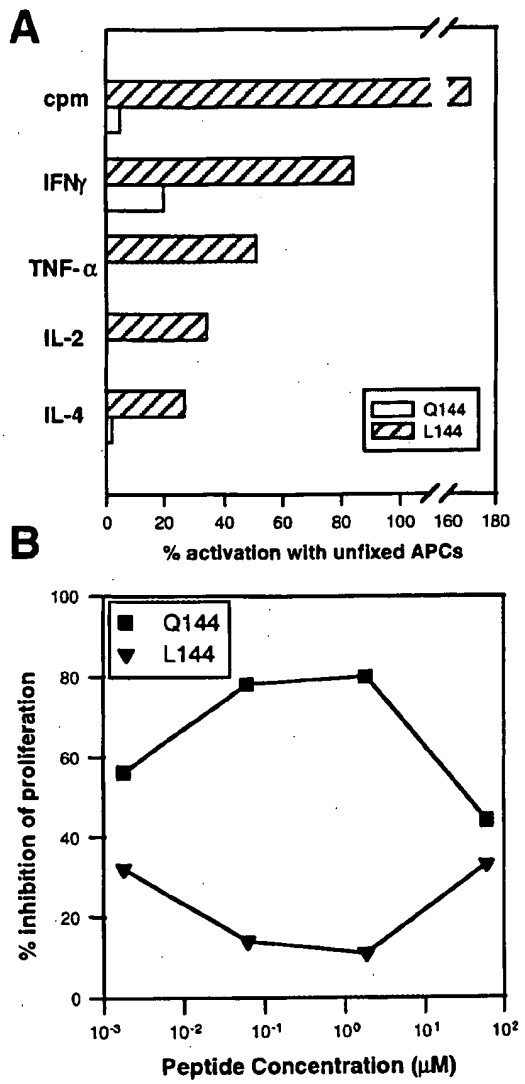

FIG. 4. Q1.1B6 is less costimulation dependent when activated with L144 compared with Q144. (*A*) Syngeneic splenic APCs were irradiated and an aliquot of cells was fixed with ECDI. Washed ECDI-fixed and unfixed-APCs were used to activate the Q1.1B6 T cell clone in a proliferation assay with 100 μg/ml of the L144 or Q144 peptides. The data are the mean of four experiments. (*B*) The Q1.1B6 was activated by syngeneic splenic APCs in the presence of human CTLA4-Ig or a control fusion protein at a concentration of 10–25 μg/ml, to determine percent inhibition. The data are the mean of two experiments.

shows that this behavior is not restricted to Q1.1B6 but can also be observed with other T cell clones.

DISCUSSION

APLs have been shown to mediate a number of different functional outcomes. These outcomes include the dominant-negative effects of T cell antagonism (12), peptide induced T cell anergy (13), and the partial activation (partial agonist) states demonstrated by the initiation of cytokine secretion in the absence of proliferation (14, 15). All these outcomes are likely to be caused by a suboptimal interaction between TCR and peptide major histocompatibility complex (MHC). By using ligands with different MHC binding affinities, it has been possible to demonstrate that increased peptide/MHC density on APCs can lead to signals which induce Th1 cytokines (30,

Table 2. T cell clones from several clonings show heteroclitic responses associated with changes in their cytokine profile

Clone	Immunizing antigen	Activating antigen	Cytokine concentration (pg/ml) IFN-γ	IL-2	IL-4
LR.1B2	L144/R147	L144/R147	<100	370	>3,200
		L144	**2,500**	**4,250**	>3,200
LR.1C1	L144/R147	L144/R147	<100	<50	2,710
		L144	<100	**370**	>3,200
QW.3F4	Q144	Q144	<100	70	1,560
		L144	**780**	**325**	1,910
QW.9F8	Q144	Q144	<100	<50	1,850
		A144	**200**	**1,550**	2,030

Superagonist ligands induce both IL-2 and IFN-γ in cells compared with activation with the immunizing peptide. Values > background are shown in bold.

31), and such ligands can induce the Th1 cytokine IFN-γ from a Th0 T cell clone. By using human T cell clones, superagonist ligands that induce heteroclitic proliferative responses from autoreactive T cells with changes in the patterns of signaling have recently been described (ref. 32; B. Hemmer and R. Martin, personal communication). We have now described a ligand with nearly identical affinity for MHC that hyperstimulates T cell activation, induces the production of cytokines not detected following activation with the cognate ligand, and has an enhanced capacity to induce high dose inhibition of proliferation. This behavior is not unique to this clone because similar responses have been seen in other clones generated by us (Table 2) and others, in independently derived murine (33) and human (B. Hemmer and R. Martin, personal communication) T cells. This leads us to propose a model in which the TCR/MHC/peptide avidity necessary to elicit a T cell response that is optimal for growth has both a lower and an upper threshold. Ligands with avidities on either side of this window are unable to initiate efficient T cell expansion either because they deliver a stimulus that is too weak or because they rapidly cause activation-induced cell death. This may serve to improve the fidelity of T cell recognition based on low affinity interactions between TCR and MHC/peptide, by providing a mechanism to neutralize the effects of high-avidity cross-reactive interactions in the periphery.

Can all TCRs respond to a range of different peptides and are T cells generally cross-reactive? The evidence that this is a common phenomenon is compelling (34). Furthermore, a recent study (35) of human T cell clones by using a random peptide library approach suggests that ligands which are heteroclitic can be generated fairly readily. Therefore, in considering whether the responses of Q1.1B6 are unusual, the important question is whether TCR interaction with cognate ligand usually induces a medium strength signal into the T cell or whether other ligands more commonly induce a maximal signal, therefore greatly reducing the likelihood of the existence of superagonist ligands for the majority of T cells. Although selection for maximal signal into the T cell cannot occur by affinity maturation of the TCR, it is possible that "affinity selection" of the T cell response can occur at the population level. The profoundly restricted T cell response to pigeon cytochrome *c* in B10.BR mice (36) may be an example of such a process. If foreign antigens do preferentially select high avidity/high signal strength T cells, then the existence of T cells with receptors that have potential superagonist ligands in the environment may be restricted to clones that have lower avidity interactions with their cognate ligands. This type of low avidity interaction is believed to characterize autoreactive TCRs, because high avidity autoreactive TCRs are deleted from the repertoire by negative thymic selection (37, 38). Therefore, low avidity autoreactive T cells may be the cells

most likely to encounter a superagonist ligand in the form of a peptide generated from a foreign organism.

These experiments also show that the induction of cytokines from Q1.1B6 is a hierarchical process, in that the concentrations of L144 necessary to elicit half-maximal production of IL-2 and TNF-α are much higher than the concentration necessary to elicit half-maximal production of IFN-γ or IL-4. However, the enormous amounts of Q144 theoretically necessary to induce the same responses as L144, and the likely upper limit on the number of class II molecules that can be loaded (39), lead us to conclude that in functional terms the two ligands produce phenotypically different responses over a wide range of antigen concentrations. Thus at a particular antigen concentration the same T cell, stimulated by different ligands, can produce a dramatically different cytokine milieu. This may effect the differentiation of naive T cells, leading to Th2 or Th1 responses depending on the activating ligand. It might also alter the responses of memory T cells and therefore may be important for initiating autoimmune reactions. For example, a self-reactive Th2 cell, activated by a cross-reactive viral superagonist peptide, might undergo a change in phenotype and become an autoaggressive Th1 cell that could traffic to a target organ and trigger an inflammatory reaction.

The less stringent requirements for costimulation that Q1.1B6 activated by L144 demonstrates may be particularly important for the initiation of autoreactivity. In organ specific autoimmunity, self-antigens may commonly be presented on nonprofessional APCs expressing MHC/peptide but no costimulatory molecules (29). If during an acute infection nonprofessional APCs within a target organ present peptides that act as superagonists for autoreactive T cells, cells that normally would be rendered anergic may be activated. The release of pro-inflammatory cytokines would subsequently up-regulate costimulation and lead to the recruitment of autoantigen specific T cells from the pool of circulating precursor T lymphocytes.

In summary, self-peptides may not act as superagonists because the self-reactive population bearing high-affinity TCR is deleted during thymic ontogeny. Superagonist ligands are more likely to be generated during infection at which time they could alter T cell differentiation, affecting the regulatory immune mechanisms that maintain peripheral tolerance, and induce autoimmunity.

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Immunology

Direct visualization of antigen-specific T cells: HTLV-1 Tax11–19-specific CD8$^+$ T cells are activated in peripheral blood and accumulate in cerebrospinal fluid from HAM/TSP patients

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ABSTRACT **Human T lymphotropic virus type 1 (HTLV-1) -associated myelopathy/tropic spastic paraparesis is a demyelinating inflammatory neurologic disease associated with HTLV-1 infection. HTLV-1 Tax11–19-specific cytotoxic T cells have been isolated from HLA-A2-positive patients. We have used a peptide-loaded soluble HLA-A2–Ig complex to directly visualize HTLV-1 Tax11–19-specific T cells from peripheral blood and cerebrospinal fluid without *in vitro* stimulation. Five of six HTLV-1-associated myelopathy/tropic spastic paraparesis patients carried a significant number (up to 13.87%) of CD8$^+$ lymphocytes specific for the HTLV-1 Tax11–19 peptide in their peripheral blood, which were not found in healthy controls. Simultaneous comparison of peripheral blood and cerebrospinal fluid from one patient revealed 2.5-fold more Tax11–19-specific T cells in the cerebrospinal fluid (23.7% vs. 9.4% in peripheral blood lymphocyte). Tax11–19-specific T cells were seen consistently over a 9-yr time course in one patient as far as 19 yrs after the onset of clinical symptoms. Further analysis of HTLV-1 Tax11–19-specific CD8$^+$ T lymphocytes in HAM/TSP patients showed different expression patterns of activation markers, intracellular TNF-α and γ-interferon depending on the severity of the disease. Thus, visualization of antigen-specific T cells demonstrates that HTLV-1 Tax11–19-specific CD8$^+$ T cells are activated, persist during the chronic phase of the disease, and accumulate in cerebrospinal fluid, showing their pivotal role in the pathogenesis of this neurologic disease.**

Human T lymphotropic virus type 1 (HTLV-1) is a human retrovirus. It can cause human adult T cell leukemia/lymphoma (1) and a slowly progressive demyelinating neurologic disease, HTLV-1-associated myelopathy/tropic spastic paraparesis (HAM/TSP) (2, 3). The clinical symptoms of HAM/TSP are characterized by upper motor neuron signs and mild sensory and sphincter dysfunction (3). Histopathologically, one finds thoracic spinal cord atrophy involving perivascular demyelination and axonal degeneration (4, 5). The pathophysiology of HAM/TSP is still not completely understood (6). Serum reactivity to HTLV-1 was first associated with HAM/TSP by Osame *et al.* in 1986 (3). The immunologic hallmark of patients with HAM/TSP is the spontaneous proliferation of peripheral blood lymphocytes (PBLs) *in vitro* (7–9).

It has been previously demonstrated that circulating CD8$^+$ cytotoxic T lymphocytes (CTLs) in patients with HAM/TSP react against HTLV-1 protein products (10), and an immunodominant HLA-A2-restricted epitope (HTLV-1 Tax11–19) has been well characterized (11). HTLV-1 Tax11–19-specific CTL precursor frequency was estimated by limiting dilution analysis in the range of 1:75 to 1:320 CD8$^+$ lymphocytes (12) in peripheral blood. Tax-specific CTLs were also found in cerebrospinal fluid (CSF) (12, 13) and HTLV-1-specific clones could be generated *in vitro* from a spinal cord lesion that was obtained from a HAM/TSP patient (14). Immunohistochemical analysis of affected spinal cord lesions in the early stage of the disease from patients with HAM/TSP reveals the presence of infiltrating CD4$^+$ and CD8$^+$ lymphocytes, in which the CD8$^+$ population predominates with duration of the disease (15). Moreover, an increase in activated lymphocytes has been shown in PBLs and CSF (16, 17). Recently, we have been able to demonstrate that peripheral CD8$^+$ T lymphocytes produce interleukin (IL) 2, γ-interferon (IFN-γ), and tumor necrosis factor α (TNF-α) in HAM/TSP patients (18). Collectively, this evidence has supported the view that virus-specific CD8$^+$ T lymphocytes may play a critical role in the immunopathogenesis of HAM/TSP.

One major limitation in these studies has been the inability to identify HTLV-1-specific CD8$^+$ T cells directly from biological samples not only to quantitate the actual number of antigen-specific T cells *in vivo* but also to further characterize the antigen-specific population of T lymphocytes without *in vitro* amplification. Recently, tetrameric major histocompatibility complex (MHC) –peptide complex crosslinked by streptavidin were shown to bind stably to antigen-specific T cells based on the increased avidity afforded by polyvalency (19). Based on this avidity principal, we have developed divalent MHC class I constructs using Ig as a scaffold (20) and used these to elucidate the role of antigen-specific CD8$^+$ T cells in different human immunologic diseases such as HAM/TSP.

Here, we have analyzed HTLV-1 Tax11–19-specific, HLA-A2$^+$-restricted CD8$^+$ T lymphocytes directly from peripheral blood and CSF using peptide-loaded divalent HLA-A2/Ig chimeras. Flow cytometric analysis showed that peptide-loaded HLA-A2/Ig specifically stained antigen-specific T cells. A surprisingly high frequency of HTLV-1 Tax11–19-specific CD8$^+$ T cells were seen in the peripheral blood of patients with HAM/TSP and a selective enrichment of these cells were seen in the CSF from a HAM/TSP patient. Tax11–19-specific CD8$^+$ T cells are activated *in vivo* in HAM/TSP and express proinflammatory cytokines. Finally, HTLV-1 Tax11–19 specific CD8$^+$ T cells persist at high numbers over

Abbreviations: HTLV-1, human T lymphotropic virus 1; HAM/TSP, HTLV-1-associated myelopathy/tropic spastic paraparesis; PBL, peripheral blood lymphocyte; CTL, cytotoxic T lymphocyte; CSF cerebrospinal fluid; IL, interleukin; IFN-γ, γ-interferon; TNF-α, tumor necrosis factor α; FITC, fluorescein isothiocyanate; PE, phycoerythrin; LDA, limiting dilution analysis; MHC, major histocompatibility complex.

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a period of 9 yrs during the chronic phase of the disease even 19 yrs after the first symptoms of the disease.

MATERIALS AND METHODS

Study Subjects and Specimens. Clinical characteristics of patients with HAM/TSP and asymptomatic HTLV-1-infected individuals as well as HLA type were described previously (12). HTLV-1 infection was confirmed by Western blot in serum of HAM/TSP patients and asymptomatic carriers. The diagnosis of HAM/TSP was determined according to W.H.O. criteria using their neurological symptoms and serological testing of HTLV-1. PBLs were prepared by Ficoll–Hypaque centrifugation and stored in liquid nitrogen until use. Histocompatibility typing was performed on PBLs or Epstein–Barr virus-transformed lymphoblastoid cell lines by the Tissue Typing Laboratory at the National Institutes of Health Clinical Center. Some of the healthy HLA-A2-uninfected controls were tested for HLA-A2 expression by fluorescence-activated cell-sorting analysis using a panel of HLA-A2-specific mAbs: MA2.1, BB7.2, and PA2.1 (American Type Culture Collection).

Peptides. Peptides HTLV-1 Tax11–19 (LLFGYPVYV), influenza virus A M158–66 (GILGFVFTL), and p17 Gag77–85 (SLYNTVATL) were synthesized and HPLC purified at the Colorado State University Macromolecular Resources (Fort Collins, CO). Identity of the peptides was confirmed by mass spectrometry measurement.

Cloning the Construct and Protein Expression. Using oligonucleotide-directed PCR, we introduced a 5′ *Mlu*I and a 3′ *Not*I site (sites underlined) into the extracellular domain (α1–α3) of HLA-A2 cDNA (kindly provided by P. F. Robbins, National Cancer Institute, National Institutes of Health, Bethesda, MD) using the following primer pair: 5′-GATACGCGTTGGGCTCTCACTCCATGAG-3′ and 5′-CAGTCGATGCGGCCGCCCATCTCAGGGTGAGGGGCT-3′. After PCR amplification the α1–α3 region of HLA-A2 was sequenced and directional cloned in exchange for H-2K^b into the previously described pX/Ig vector (20). Subsequently, the construct was cotransfected with DNA encoding the chimeric HLA-A2/Ig protein and the human β_2-microglobulin by electroporation into J558L cells. Transfectants were screened for secretion of the chimeric protein by ELISA as described previously (20). ELISA plates were coated with BB7.2, an antibody specific for peptide-loaded conformations of HLA-A2 or a goat anti-mouse IgG–Fc antibody (10 μg/ml; Cappel). High secretors were picked and grown in Hybridoma-SFM (GIBCO/BRL). HLA-A2/Ig secretion from the transfected J558L was confirmed by SDS/PAGE. The chimeric protein was harvested from cell supernatant and was concentrated with Centriflo-membrane cones (Amicon). HLA-A2/Ig was loaded with 660-fold molar excess of peptide for 10–14 days before fluorescence-activated cell-sorting analysis. Three micrograms of protein was used for staining of 1×10^6 cells.

Flow Cytometric Analysis. Murine monoclonal anti-human CD8–fluorescein isothiocyanate (FITC; Sigma), anti-CD4-phycoerythrin (PE; Becton Dickinson), anti-CD8-Tri (Caltag Laboratories, Burlingame, CA), anti-HLA-DR (PharMingen) were used to detect cell surface molecules of lymphocytes. PK1.36–FITC was used as an isotype-matched control for anti-HLA-DR–FITC. HLA-A2/Ig bound onto the cell surface was detected using goat anti-mouse IgG1–PE or a goat anti-mouse IgG1–Tri (Caltag). Monoclonal anti-TNF-α–FITC, anti-IFN-γ–FITC, or isotype-matched IgG1–FITC antibodies (PharMingen) were used for intracellular cytokine staining. PBLs (1×10^6) were incubated with peptide-loaded HLA-A2/Ig on ice followed by PE or Tri-conjugated goat anti-mouse IgG1–PE. Intracellular staining was performed according to the manufacturer's instructions, with slight modifications (21), after a 4-h incubation at 37°C followed by a 10-h incubation in the presence of 10 μg/ml brefeldin A (Sigma). Three-color fluorometric analysis was carried out on a FACScan (Becton Dickinson). Lymphocytes were gated on forward and side scatter. One $\times 10^5$ (2×10^5 for three-color analysis) gated events were analyzed using CELLQuest software (Becton Dickinson).

PBLs from all HLA-A2-positive patients have been stained at least twice with similar results.

RESULTS

Peptide-Loaded HLA-A2/Ig Binds Specifically HLA-A2-Restricted CTLs. We have engineered a divalent HLA-A2/Ig molecule using Ig as a scaffold that can be loaded with various peptides. Peptide-loaded HLA-A2/Ig was specific in its interaction with HLA-A2-restricted CTL clones analyzed by flow cytometry. Thus, Tax-A2/Ig was specifically reactive with a HLA-A2-restricted, HTLV-1 Tax11–19-specific CTL clone, A6 (22). Mean channel fluorescence of A6 cells stained with HTLV-1 Tax11–19-loaded HLA-A2/Ig (Tax-A2/Ig) was 426, whereas mean channel fluorescence of A6 cells stained with the control Gag-A2/Ig complex was only 13 and almost identical to staining controls using no chimeric protein, unloaded HLA-A2/Ig or M1-loaded HLA-A2/Ig (Fig. 1*A*). In complementary experiments, Gag-A2/Ig specifically stained the HIV p17 Gag77–85-specific clone SL09 (23) (Fig. 1*B*), whereas staining with Tax-A2/Ig was virtually identical to unloaded HLA-A2/Ig, M1-loaded HLA-A2/Ig, or no HLA-A2/Ig.

Visualization of Antigen-Specific T Lymphocytes in Peripheral Blood of Patients with HAM/TSP. HTLV-1 Tax11–19-specific CTL activity has been demonstrated directly in PBLs from HAM/TSP patients (10). In HLA-A2$^+$ patients, HTLV-1-specific reactivity is predominately directed against the

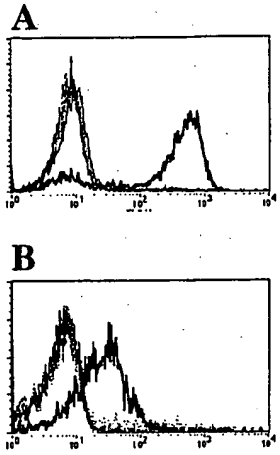

FIG. 1. Peptide-loaded HLA-A2/Ig specifically stains HTLV-1 Tax11–19 or HIV p17 Gag77–85-specific T cell clones. (*A*) Fluorescence-activated cell-sorting analysis was carried out using A6 cells (22), specific for the immunodominant HLA-A2-restricted Tax epitope Tax11–19. HTLV-1 Tax11–19-loaded HLA-A2/IgG stably bound on the cell surface and was detected using PE-labeled goat anti-mouse Ig (heavy line). Gag-A2/Ig was used as an irrelevant control (thin line) as well as no HLA-A2/Ig, unloaded HLA-A2/Ig, and M1-loaded HLA-A2/Ig (dotted and stripped lines overlapping). (*B*) T cells specific for the HLA-A2-restricted HIV p17 Gag77–85 epitope were stained to demonstrate the peptide specificity of peptide-loaded HLA-A2/Ig. Gag77–85-loaded HLA-A2/IgG was stably bound on the cell surface and detected using PE-labeled goat anti-mouse Ig (heavy line). HTLV-1 Tax11–19-loaded HLA-A2/Ig as well as no peptide loaded, M1-loaded HLA-A2/Ig, and no HLA-A2/Ig were used as an irrelevant control and stained virtually identical (overlapping thin, dotted, and stripped lines).

HTLV-1 Tax11–19 epitope (11). Previous estimates of precursor frequencies of HTLV-1 Tax11–19-specific CTLs using limiting dilution analysis (LDA) gave values in the range of 1:75–1:320 $CD8^+$ T cells. To directly quantitate the HTLV-1 Tax11–19-specific $CD8^+$ population in peripheral blood of HAM/TSP patients, we performed two-dimensional flow cytometric analysis of cells using Tax-A2/Ig and anti-$CD8^+$ (Fig. 2). PBLs from patients with HAM/TSP showed a significant high number of HTLV-1 Tax11–19-specific $CD8^+$ T lymphocytes. In one patient the frequency of Tax11–19-specific cells was as high as 13.8% (Figs. 2 and 3). The percentage of Tax-A2/Ig-specific cells ranged between 0.64 and 13.87% of $CD8^+$ T cells with values >2% for four of the six HAM/TSP patients (Fig. 3). In one of the six HLA-A2-positive HAM/TSP patients, no Tax-A2/Ig-reactive cells were detected. However, this same patient's PBLs also did not lyse Tax protein-expressing, autologous, Epstein–Barr virus-transformed B cells (our unpublished data). Moreover, we were able to detect Tax-specific T cells with Tax-A2/Ig in every patient sample that lysed Tax-expressing, $HLA\text{-}A2^+$ target cells.

We also analyzed peripheral blood from two $HLA\text{-}A2^+$ HTLV-1 carriers who did not have HAM/TSP (Fig. 3). One of the two HTLV-1 carriers had no detectable HTLV-1 Tax11–19-specific T cells, whereas the other contained a low but reproducibly detectable number of $CD8^+$ T cells that stained with Tax-A2/Ig (0.33%). To confirm the specificity of staining of peripheral blood samples with these reagents and their relationship to the disease, we analyzed PBLs from HAM/TSP patients who do not carry the *HLA-A2* allele (Fig. 3), uninfected healthy $HLA\text{-}A2^+$ donors (Fig. 3), and a HIV-1-infected $HLA\text{-}A2^+$ individual (Fig. 2). Less then 0.1% of $CD8^+$ T cells from all of these samples stained positively with the Tax-A2/Ig. However, a significant number (3.03%) of the $CD8^+$ T lymphocytes from the HIV-infected individual was stained using Gag-A2/Ig. Collectively, these results demonstrate that the presence of high numbers of Tax-A2/Ig-staining $CD8^+$ cells is specific for $HTLV\text{-}1^+$ $HLA\text{-}A2^+$ individuals with HAM/TSP.

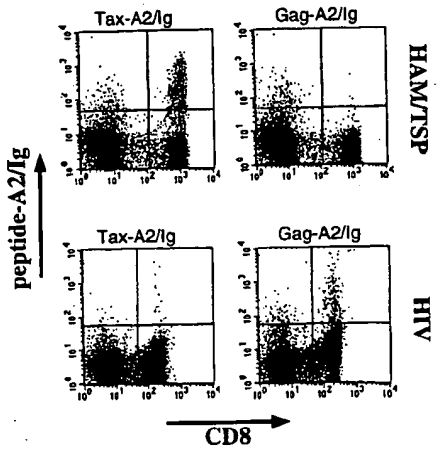

FIG. 2. Peptide-loaded HLA-A2/Ig can be used to visualize antigen-specific $CD8^+$ T cells in peripheral blood. Two-color flow cytometry was performed using Tax-A2/Ig or Gag-A2/Ig and mouse anti-human CD8-FITC. PE-labeled goat anti-mouse IgG1 mAb was used to detect the peptide-A2/Ig complex. Frozen PBLs from a HAM/TSP patient (donor H1) were examined. Four and three-tenths percent of the PBLs from the HAM/TSP patient stained positive with anti-human CD8-FITC and Tax-A2/Ig. This represents 10.49% of all $CD8^+$ cells. No significant signal was seen using Gag-A2/Ig (<0.1%). PBLs from a $HLA\text{-}A2^+$ HIV-1-infected individual showed 3% Gag-A2/Ig-positive $CD8^+$ T lymphocytes.

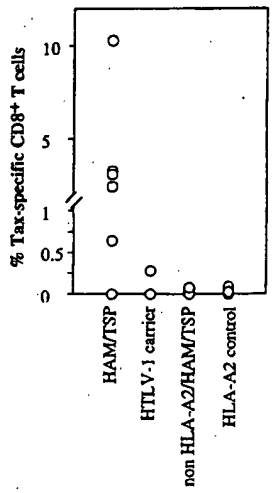

FIG. 3. HAM/TSP patients carry high numbers of HTLV-1 Tax11–19-specific $CD8^+$ cells in their PBLs. Presented is the percentage of HTLV-1 Tax11–19-specific, $CD8^+$ T cells from $HLA\text{-}A2^+$ HAM/TSP patients, HTLV-1-infected $HLA\text{-}A2^+$ carriers, $HLA\text{-}A2^-$ HAM/TSP patients, and healthy individuals. Between 0.64 and 10.25% of the $CD8^+$ cells from HAM/TSP patients are specific for the HTLV-1 Tax11–19 peptide. One of the analyzed HTLV-1-infected donors had 0.33% Tax-specific $CD8^+$ cells, whereas no significant number of Tax-specific $CD8^+$ cells were found in non-HLA-A2 HAM/TSP donors or $HLA\text{-}A2^+$ healthy individuals. Positive samples were stained at least twice.

Visualization of Antigen-Specific T Lymphocytes in CSF of Patients with HAM/TSP. To corroborate the significance of HTLV-1-specific CTLs for the pathogenesis of this demyelinating disease, we analyzed lymphocytes from CSF of a patient with HAM/TSP (H1) and compared them with the T cells in his peripheral blood from the same day. Of the $CD8^+$ lymphocyte population in CSF, 23.7% was Tax-A2/Ig specific. In contrast, we detected 9.4% Tax-A2/Ig-specific $CD8^+$ T cells in the peripheral blood (Fig. 4*A*). Interestingly, we found the same number of $CD4^+$ T cells in peripheral blood and CSF (44% in the PBLs and 48% in the CSF) as well as an increased number in total $CD8^+$ T cells in the CSF (42%) compared with the peripheral blood (29%) (Fig. 4*B*).

HTLV-1 Tax11–19-Specific T Cells in Patients with HAM/TSP Are Activated. The ability to directly stain HTLV-1 Tax11–19-specific T lymphocytes provides the opportunity to analyze their state of activation. If HTLV-1-specific $CD8^+$ T cells indeed mediate the immunopathogenesis of HAM/TSP, a significant proportion of them might be expected to be activated. Since activated human T cells express elevated levels of MHC class II molecules, we further characterized the HTLV-1 Tax11–19-specific T cells from HAM/TSP patients for HLA-DR expression by multicolor flow cytometry.

Tax-A2/Ig-reactive $CD8^+$ T cells from the three HAM/TSP patients had significantly increased expression of DR (Fig. 5). In two of three patients, the level of DR expression was virtually identical to that seen in control phytohemagglutinin-stimulated PBLs (Fig. 5*A* and *B*, Tax-A2/Ig- reactive-specific $CD8^+$ T cells from patients, to *E*, activated human PBLs). Similar results were obtained for IL-2 receptor expression (data not shown). Of particular interest, one patient, H6, demonstrated a significantly lower population of DR^+, Tax-specific $CD8^+$ cells (Fig. 5*C*). This individual, the spouse of a HAM/TSP patient, was originally thought to be an asymptomatic carrier, since no neurologic symptoms were reported. However, on a physical examination a hyperreflexia in the lower extremities and extensor plantar responses were observed, indicative of corticospinal tract lesion(s).

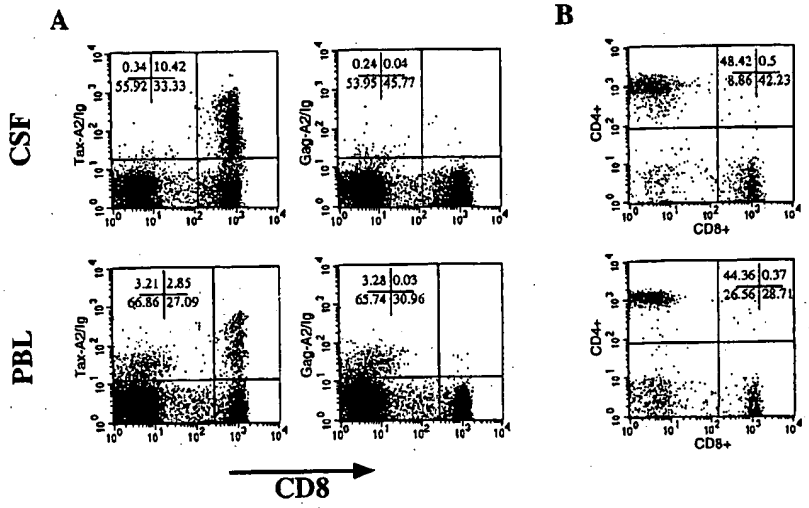

FIG. 4. HTLV-1 Tax11–19-specific CD8+ T cells accumulate in CSF. CSF and peripheral blood were obtained from a HAM/TSP patient (H1) the same day and analyzed using Tax-A2/Ig. Cells (9×10^4) were obtained from CSF and stained using Tax/A2-Ig and Gag/A2-Ig in combination with mouse anti-human CD8-FITC as described in Fig. 3. Freshly obtained, Ficoll-purified PBLs were analyzed in parallel. Of the CD8+ T cells in CSF, 23.7% were Tax specific and only 9.4% of the CD8+ T cells from peripheral blood. No significant signal was seen using Gag-A2/Ig (<0.1%) (*A*). CD4/CD8 analysis showed that the ratio of CD4:CD8 was 1.1 in the CSF and 1.5 in the PBLs due to a higher number of CD8+ T cells in CSF (*B*).

Inflammatory cytokines are considered critical mediators of the central nervous system immunopathology in many autoimmune models. Elevated levels of several proinflammatory cytokines have been detected in the serum or CSF of HAM/TSP patients (16, 17). Therefore, it is of particular interest to analyze Tax-specific CD8+ cells from HAM/TSP patients for the presence of relevant intracellular cytokines. Recently, we have found that bulk CD8+ T cells from HAM/TSP patients demonstrated significant expression of cytokines such as TNF-α, IFN-γ, and IL-2 but no IL-4 (18). Tax-specific CD8+ T cells from patients H1 (Fig. 6), H5 (data not shown), and H6 (Fig. 6) demonstrated distinct patterns of intracellular TNF-α and IFN-γ expression. Although the HAM/TSP patients H1 and H5 displayed a high proportion of Tax-A2/Ig-specific CD8+ cells with intracellular TNF-α and IFN-γ (roughly 30%), patient H6 had a very low proportion of Tax-specific CD8+ cells expressing intercellular TNF-α and IFN-γ (2%). These results, along with HLA-DR analysis, demonstrate a disassociation between expansion of antigen-specific T cells and their activation state. They further demonstrate how the ability to use multiparameter flow cytometry to analyze the activation state of antigen-specific T cell populations may provide further insight into the relationship between state of T cell activation and disease pathogenesis.

Tax-Specific CD8+ T Cells Persist in Peripheral Blood from HAM/TSP Patients. An important aspect in understanding the pathogenesis of autoimmune diseases, such as HAM/TSP, is to understand the dynamics of antigen-specific T cell responses. We therefore compared the numbers of Tax-specific, CD8+ T cells from a HAM/TSP patient (H1) who had serial

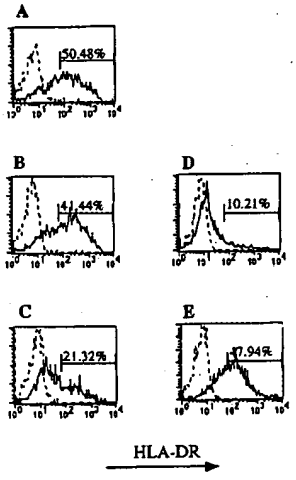

FIG. 5. Tax-specific CD8+ cells from HAM/TSP patients express cell surface activation marker HLA-DR. Using three-color flow cytometry, Tax-specific CD8+ cells were gated and analyzed for HLA-DR expression using mouse anti-human HLA-DR-FITC (solid line) and an isotype-matched irrelevant mouse IgG2a-FITC control (dotted line). Forty-one to 50% of the Tax-specific CD8+ T cells from two different symptomatic HAM/TSP patients stain positive for HLA-DR expression (*A* and *B*) in contrast to only 21% from patient H6 (*C*), who has a very mild form of the disease. Nonstimulated (*D*) and phytohemagglutinin-stimulated (*E*) CD8+ PBLs from a healthy donor served as staining controls.

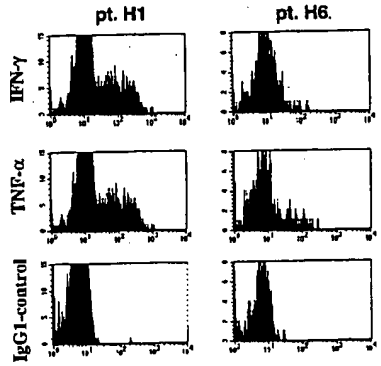

FIG. 6. Tax-specific CD8+ cells from HAM/TSP patients express intracellular IFN-γ and TNF-α. Using three-color flow cytometry, Tax-specific CD8+ cells were gated and analyzed for intracellular cytokine expression as described in *Materials and Methods*. Using mouse anti-human IFN-γ–PE, mouse anti-human TNF-α–PE, and an isotype-matched irrelevant mouse IgG1–PE control, 28% of the Tax-specific CD8+ cells from H1 (left panel) expressed intracellular IFN-γ and 29% expressed TNF-α; however, only 8% of the Tax-specific CD8+ cells from H6 (right panel) expressed intracellular IFN-γ and TNF-α.

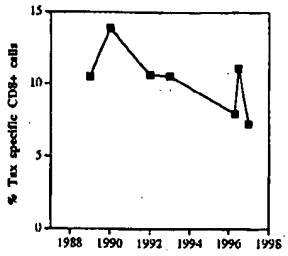

FIG. 7. High levels of HTLV-1 Tax-specific $CD8^+$ T lymphocytes persist during HAM/TSP. PBL samples obtained between 1989 and 1998 from patient H1 were analyzed for Tax-specific $CD8^+$ cells. Between 7.24 and 13.87% specific $CD8^+$ T cells were detected in the frozen PBL samples.

PBLs drawn between 1989 and 1998. This patient was initially diagnosed in 1979. Interestingly, even 10 yrs after diagnosis, this patient had about 10% Tax-specific $CD8^+$ T cells which did not change significantly over the next 8 yr (Fig. 7). Thus, in this patient pathogenic Tax-specific $CD8^+$ T cells are sustained at high levels even after 19 yrs of active disease.

DISCUSSION

In the present study, we demonstrate that divalent peptide loaded HLA-A2/Ig can be used to directly stain antigen-specific, HLA-A2-restricted T cells in peripheral blood. Initially, we chose the most common HLA class I allele (24) because many HLA-A2-restricted antigens have been identified for human viral infections, including HIV (25), autoimmune diseases, and cancer (26). However, we have already successfully used this approach for other MHC class I molecules (data not shown). This class of reagents represents a new tool for understanding a variety of immunologic diseases. The observation that a divalent MHC/Ig chimeric protein stably binds to its cognate T cell receptor is surprising since the monovalent peptide–MHC complex dissociates rapidly from complexes with TCR with a half-life of <1 min (27), and the affinity of the T cell receptor for peptide–MHC complexes is relatively low ($\approx 10^{-5}$ μM) (28–30). Stable binding of the dimeric HLA-A2/Ig complex to cognate T cells is based on two features related to the Ig scaffold. First, the divalent nature of the complex provides an increase in avidity. An increase in avidity afforded by polyvalence has also been demonstrated using tetrameric peptide–MHC complexes crosslinked by streptavidin (19). Second, it is likely that the unique flexibility of the Ig hinge region promotes maximal avidity enhancement with our divalent construct.

Using the Tax-A2/Ig to detect antigen-specific T cells, we have been able to identify surprisingly large numbers of Tax-specific $CD8^+$ T cells in the peripheral blood of the majority of HAM/TSP patients without *in vitro* amplification. These high numbers apparently do not represent acute spikes of T cell expansion since consistently high numbers of cells were observed in at least one patient (Fig. 7) on eight different analyses over a 9-yr period. Finally, similar numbers of Tax-specific $CD8^+$ T cells over time were also found in blood samples from other patients that were taken at different time points (data not shown).

The ability to simultaneously quantitate Tax-specific $CD8^+$ T cells in both PBLs and CSF has enabled us to directly analyze the role of these cells at the site of pathology. Since HAM/TSP is a demyelinating neurological disease in which $CD4^+$ and $CD8^+$ T cells infiltrate the spinal cord (14), analysis of T lymphocytes from CSF is pivotal. We found a higher number of Tax-specific $CD8^+$ T cells in the CSF than in peripheral blood, suggesting that these antigen-specific T cells accumulate in the central nervous system. Accumulation of Tax11–19-specific $CD8^+$ T cells at the pathological site, the central nervous system, could be due to either migration and selective accumulation of antigen-specific T cells in the central nervous system or selective expansion of these cells in the central nervous system. In either event, selective enrichment of these cells strongly suggests that these cells are directly involved in the pathogenesis of HAM/TSP.

Quantitation of HTLV-1 Tax11–19-specific T cells in peripheral blood with peptide-loaded HLA-A2/Ig demonstrates that previous precursor frequency analysis of Tax-specific $CD8^+$ CTLs by LDA may have significantly underestimated the actual number of Tax-specific T cells (12). We were able to show that the number of Tax-specific $CD8^+$ T cells assessed by Tax-A2/Ig staining was roughly 10- to 30-fold higher than previously estimated by LDA (12). The accuracy of LDA is probably compromised by the fact that it depends on the ability to expand individual cells *in vitro* to numbers large enough to be detected by functional tests such as chromium release (31–34). The frequencies determined by direct staining with Tax-A2/Ig are not due to nonspecific binding. Tax-A2/Ig positively staining $CD8^+$ cells were virtually undetectable in $HTLV\text{-}1^-$ individuals, in $HLA\text{-}A2^-$ patients with HAM/TSP, and in control HLA-A2 patients infected with a different retrovirus, HIV, where a significant number of cells were specific for p17 Gag77–85 when stained with p17 Gag77–85-loaded A2/Ig.

It is still unknown why some HTLV-1-infected individuals develop an inflammatory neurologic disease and what pathophysiologic mechanisms lead to the development of HAM/TSP. In many models HTLV-1-specific $CD8^+$ lymphocytes are considered to play a crucial role, although it remains unknown which cells in the central nervous system are preferential targets for these cells (35–40). The ability to directly visualize Tax-specific T cells in peripheral blood and the characterization of this cell population has provided new insights into the pathogenesis of HAM/TSP that could also be of relevance to other autoimmune diseases. We were able to clearly demonstrate for the first time that circulating Tax-specific, $CD8^+$ T lymphocytes are activated as measured by expression of HLA-DR staining in HAM/TSP patients. We also further characterized these antigen-specific $CD8^+$ T lymphocytes for cytokine production since we recently found that bulk $CD8^+$ T lymphocytes from HAM/TSP patients release cytokines dependent on the presence of either HTLV-1-infected $CD4^+$ T lymphocytes or peptide-pulsed allogeneic $HLA\text{-}A2^+$ B lymphocytes (18). Three-color analysis ($CD8^+$ vs. Tax-A2/Ig vs. TNF-α or IFN-γ) of PBLs from patients H1 and H5 revealed a number of interesting features. Although a very large proportion of $CD8^+$ Tax-$A2/Ig^+$ cells expressed intracellular IFN-γ and TNF-α (28 and 29%, respectively), the majority were negative for these cytokines, suggesting that circulating Tax-specific $CD8^+$ cells are not uniformly activated. The cytokine-negative cells may represent a dormant cell population not actively participating in disease pathogenesis.

Although the number of analyzed individuals is clearly too small to draw any definite conclusions, the observation that Tax-specific T cells from H6 showed lower expression of intracellular IFN-γ and TNF-α as well as less MHC class II expression than other HAM/TSP patients may be significant. This individual has a very mild form of the disease (no clinical symptoms and neurologic deficits limited to hyperreflexia in the lower extremities and extensor plantar responses indicative of a corticospinal tract lesion). One might therefore speculate that this individual has not developed any additional symptoms and still might be at an very early stage of the disease due to the lack of activated HTLV-1 Tax11–19-specific $CD8^+$ T lymphocytes. Characterization of HTLV-1-specific lymphocytes from a larger cohort of patients to determine which parameters of T cell activation and cytokine expression cor-

relate the best with disease severity and progression will further elucidate the role of activated HTLV-1 Tax11–19-specific CD8+ T lymphocytes in the pathogenesis of HAM/TSP.

In addition to using peptide-loaded HLA-A2/Ig to analyze antigen-specific T cell populations, the Ig scaffold of the HLA-A2 Ig chimera could provide for a variety of other applications, including targeting of antigen-specific T cells *in vivo*. The diversity of *in vivo* biological effects mediated by the different Fc regions which can be inserted onto this molecule allow for its application in targeting antigen-specific T cells *in vivo*, either for amplification of antigen-specific T cells as a vaccine or for elimination of pathogenic T cells in diseases such as HAM/TSP as well as classical autoimmune diseases. The high number of HTLV-1 Tax11–19-specific CD8+ T cells in HLA-A2+ HAM/TSP patients suggests the HTLV-1 Tax11–19 may represent the immunodominant epitope in this pathogenic immune response. Thus, soluble divalent MHC/Ig could also have therapeutic application in targeting and eliminating HTLV-1-specific CD8+ T lymphocytes in HAM/TSP.

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T lymphocytes from human atherosclerotic plaques recognize oxidized low density lipoprotein

(antigen/atherosclerosis/immune response/oxidation)

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ABSTRACT **Atherosclerosis, an underlying cause of myocardial infarction, stroke, and other cardiovascular diseases, consists of focal plaques characterized by cholesterol deposition, fibrosis, and inflammation. The presence of activated T lymphocytes and macrophages and high expression of HLA class II molecules are indicative of a local immunologic activation in the atherosclerotic plaque, but the antigen(s) involved has not yet been identified. We established T-cell clones from human atherosclerotic plaques using polyclonal mitogens as stimuli and exposed the clones to potential antigens in the presence of autologous monocytes as antigen-presenting cells. Four of the 27 CD4⁺ clones responded to oxidized low density lipoprotein (oxLDL) by proliferation and cytokine secretion; this response was dependent on autologous antigen-presenting cells and restricted by HLA-DR. All clones that responded to oxLDL secreted interferon γ upon activation, but only one produced interleukin 4, suggesting that the response to oxLDL results in immune activation and inflammation but may not be a strong stimulus to antibody production. No significant response to oxLDL could be detected in CD4⁺ T-cell clones derived from the peripheral blood of the same individuals. Together, the present data suggest that the inflammatory infiltrate in the atherosclerotic plaque is involved in a T-cell-dependent, autoimmune response to oxLDL.**

Atherosclerosis, an underlying cause of myocardial infarction, stroke, and other cardiovascular diseases, consists of focal lesions of the arterial intima which are characterized by cholesterol deposition, fibrosis, and inflammation (1). These lesions begin as local infiltrates of monocyte-derived macrophages, T lymphocytes, and lipoproteins (1–3). Experimental data suggest that local endothelial expression of the vascular cell adhesion molecule 1 (4) and chemotactic stimulation by oxidized low density lipoprotein (oxLDL; ref. 5) may be important for the formation of this early lesion. During the subsequent progression of the lesion, macrophages are transformed into lipid-laden foam cells, presumably by uptake of oxLDL (2, 6, 7), and smooth muscle cells migrate into the lesion to form a fibrous cap around the lipid-rich core (1). Activated macrophages and T lymphocytes may, by means of their cytokine secretion, regulate foam cell transformation, smooth muscle proliferation, and the generation of free oxygen radicals (3).

Low density lipoprotein (LDL) is modified by oxidation in macrophage-rich tissues and large amounts of modified LDL can be detected in plaques (8–10). Not only does such modified LDL stimulate T-cell migration (5), but it is immunogenic and induces antibody production (9–11). In fact, the systemic, humoral immune response to oxidation-generated epitopes on LDL correlates with the progression of carotid atherosclerosis (12). Immune complexes consisting of oxLDL and antibodies to oxLDL can be found in atherosclerotic lesions of rabbits and humans (13). We therefore speculated that the T cells and macrophages in the plaque may participate in an immune response to oxLDL.

Plaque T cells are phenotypically different from those of peripheral blood, since they represent a memory cell subset characterized by expression of the CD45R0 surface protein and by surface markers of late-stage activation (14). This phenotype has the capacity for secretion of several cytokines (15) and there is direct evidence for a local secretion of interferon γ (IFN-γ) in the plaque (16). In addition, HLA class II molecules are expressed by smooth muscle cells, endothelial cells, and macrophages of the plaque (17, 18). These proteins can be induced by IFN-γ, and the data therefore support the concept that activated plaque T cells regulate gene expression in surrounding cells by a paracrine, cytokine-mediated pathway (3, 19).

The presence of activated T lymphocytes and macrophages (14, 16, 17, 20–22) and extensive expression of HLA class II molecules (17) are indicative of a local immunologic activation in the atherosclerotic plaque, but the antigen(s) involved has not yet been identified. We have established T-cell clones from atherosclerotic plaques by using polyclonal mitogens as stimuli. Because oxLDL is present in atherosclerotic plaques in significant amounts and is immunogenic, we asked whether the isolated clones would respond to oxLDL in the presence of autologous monocytes. We now report that a significant proportion of the CD4⁺ clones responded to oxLDL by proliferation and cytokine secretion and that this response was dependent on autologous antigen-presenting cells and restricted by HLA-DR. These data suggest that atherosclerosis involves an autoimmune response to oxLDL.

MATERIALS AND METHODS

Isolation and Cloning of T Cells from Human Atherosclerotic Plaques. Atherosclerotic plaques were obtained from three patients undergoing carotid surgery due to transitory ischemic attacks. Cells were isolated by collagenase digestion and T lymphocytes were cloned from the cell suspension by limiting dilution (23). Mitogenic, OKT3 antibodies (Ortho Diagnostics) were used to cross-link the antigen receptor complex, recombinant interleukin 2 (IL-2, Ala-125 substituted; Amersham) was used to promote growth, and irradiated peripheral blood mononuclear cells (PBMC) were used as feeder cells to maintain the clones (23). PBMC were isolated from peripheral blood by centrifugation over Ficoll-Paque (Pharmacia). A total of 105 T-cell clones were generated from the endarterectomy material of patients NN (53 clones), RB

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Abbreviations: LDL, low density lipoprotein; oxLDL, oxidized low density lipoprotein; IFN-γ, interferon γ; PBMC, peripheral blood mononuclear cells; nLDL, native LDL; IL-*n*, interleukin *n*.

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(21 clones), and KB (31 clones) by using this technique. Similar clonal libraries were established from peripheral blood of the same individuals. Since polyclonal mitogens (OKT3 antibodies) rather than specific antigens were used to activate the T cells, the clonal libraries thus established had the potential of containing a multitude of different immunologic reactivities present among the T cells in the plaque (23).

Generation and Assay of Tetanus Toxoid-Specific T-Cell Clones. PBMC were isolated from the peripheral blood of a normal donor as described above. T cells were cloned by limiting dilution in Terasaki 60-well plates in the presence of 10^5 irradiated autologous PBMC and 25 μg of tetanus toxoid (SBL-Vaccin, Stockholm) per ml in RPMI 1640 culture medium with 10% fetal calf serum and 10% pooled human serum from healthy donors. Growing clones were stimulated to proliferate with OKT3 antibody and IL-2 as described (23). They were then subjected to proliferation assays in the presence of tetanus toxoid at 10–50 μg/ml, ovalbumin (Calbiochem–Novabiochem) at 25 μg/ml, and native or oxLDL at 10 μg/ml as stated in *Results*.

Immunophenotyping. T-cell clones were stained with fluorescent antibodies to CD3, CD4, and CD8 (monoclonal antibodies Leu-4, Leu-3a, and Leu-2, respectively, from Becton Dickinson) and analyzed in a FACScan flow cytometer (Becton Dickinson) by using the LYSYS II software package.

LDL Oxidation. LDL was isolated in the presence of 1 mM EDTA from the pooled sera of fasting human subjects by ultracentrifugation through KBr as described (24). KBr was removed by gel filtration on Sephadex G-25 M (PD-10; Pharmacia) and 200 μg of LDL per ml was oxidized by incubation with 5 mM $CuSO_4$ in 5 mM Hepes buffer containing 150 mM NaCl, 4 mM $CaCl_2$, and 2 mM $MgCl_2$ (pH 7.2) for 20 h at 37°C (25, 26). Oxidation was then inhibited by addition of butylated hydroxytoluene to a final concentration of 20 μM (26). The degree of oxidation was evaluated by an assay for thiobarbiturate-reactive substances (TBARS; refs. 25 and 27) and LDL protein was characterized by agarose electrophoresis and quantitated by Bradford's method (28). TBARS values were 16 ± 8 mmol of malondialdehyde equivalents per mg of protein for oxLDL preparations and <1 mmol/mg for native LDL (nLDL). nLDL was maintained in 20 μM butylated hydroxytoluene to prevent oxidation (11). All lipoprotein preparations were sterilized by filtration through 0.22 μm Millex GV filters before use.

Blocking Antibodies. The hybridoma L243, which produces a monoclonal antibody to human HLA-DR (29), was obtained from the American Type Culture Collection and grown in Iscove's modified Dulbecco's medium with 2 mM L-glutamine, 0.45 mM sodium pyruvate, 50 mM 2-mercaptoethanol, 0.5% Primaton RL (Sheffield Product, Kraft), and 10% fetal bovine serum. Culture in dialysis tubing (30) was used to obtain large amounts of antibody, which was purified from the medium by affinity chromatography over protein G-Sepharose (Pharmacia). The purified L243 IgG was dissolved in phosphate-buffered saline (PBS; 154 mM NaCl/10 mM sodium phosphate, pH 7.2) and used at 10 μg/ml in blocking experiments. The mouse myeloma IgG MOPC 21, which does not recognize any known determinant in human cells (31), was obtained from Sigma and used in experiments in the same way as was L243.

Antigen Presentation Assay. Venous blood samples were obtained from the patients by renewed bleedings, and PBMC were isolated as described above. $CD4^+$ T cells (10^5) were incubated with 10^5 autologous PBMC in a volume of 300 μl of RPMI 1640 medium with 10% fetal bovine serum and 10% pooled, heat-inactivated human serum in 96-well, round-bottomed, microtiter plates. The antigen-presenting PBMC were irradiated with 25 Gy from a cobalt source to eliminate their proliferative capacity before being incubated. oxLDL and/or antibodies were added to the cultures as described in *Results*, and 72 h later, 2 μCi of [^{3}H]thymidine (1 Ci = 37 GBq; Amersham) was added to each well. Nonadherent cells were harvested on glass fiber filters with an Inotech (Wohlen, Switzerland) Cell Harvester after an additional 24 h of culture. [^{3}H]Thymidine uptake was determined by liquid scintillation.

Cytokine Secretion. IFN-γ (Biosource, Camarillo, CA) and IL-4 (R & D Systems) were measured by ELISA in 100 μl of culture medium collected from each of the T-cell clones cocultured with PBMC for 72 h after being stimulated with OKT3 and IL-2.

RESULTS

Atherosclerotic plaques were obtained from three patients undergoing carotid artery surgery due to transitory ischemic attacks. Cells were isolated by collagenase digestion, and T lymphocytes were cloned from the cell suspension by limiting dilution (23). Mitogenic OKT3 antibodies were used to cross-link the antigen receptor complex, recombinant IL-2 was added to promote growth, and irradiated, autologous PBMC were used as feeder cells to maintain the clones (23). Large numbers of T-cell clones of both CD4 and CD8 phenotypes were generated from each plaque biopsy by using this technique (Table 1). Similar clonal libraries were established from the peripheral blood of the same individuals. Since polyclonal mitogens (OKT3) rather than specific antigens were used to activate the T cells, the clonal libraries thus established had the potential of containing many of the different immunologic reactivities that had been present in the T-cell population in the plaque (23).

Southern blot analysis of the organization of the T-cell receptor genes indicated that all clones displayed different gene rearrangement patterns; they were therefore of polyclonal origin (23). A phenotypic analysis was carried out by flow cytometry. All clones expressed CD3 and either CD4 or CD8, indicating that they were mature, T-cell receptor $\alpha\beta$-type T cells (23). The majority of clones in all three libraries were of the $CD4^+$ phenotype (Table 1). Since exogenous antigens are presented by major histocompatibility complex class II molecules, which are exclusively recognized by $CD4^+$ T cells, clones of this phenotype were selected for the antigen-presentation analyses.

The hypothesis that oxLDL is an important local (auto)antigen in the atherosclerotic plaque was tested by challenging our T-cell clones with oxLDL. $CD4^+$ clones were exposed to copper-oxidized LDL in the presence of irradiated, autologous PBMC that could serve as antigen-presenting cells. $CD4^+$ T cells immunospecific for oxLDL would be expected to respond to this stimulus by proliferation. Fig. 1 shows a typical positive response. The $CD4^+$ clone RB2 responded to oxLDL by a 5-fold increase in DNA synthesis compared with the level in the absence of oxLDL (Fig. 1). The response was dependent on the presence of antigen-presenting cells, since addition of oxLDL to RB2 in the absence of PBMC did not induce proliferation (Fig. 1).

Table 1. Patients and T-cell clones used in this study

		Plaque-derived clones			Peripheral blood-derived clones		
Patient	Age, yr	Total no.	$CD4^+$	Tested	Total no.	$CD4^+$	Tested
NN	70	53	40	10	47	39	7
RB	61	21	16	8	14	12	8
KB	57	31	26	9	—	—	—

Clones were established by limiting dilution using OKT3, recombinant IL-2, and autologous feeder cells. CD4 reactivity was analyzed by flow cytometry, and clones were randomly chosen for testing in antigen assays.

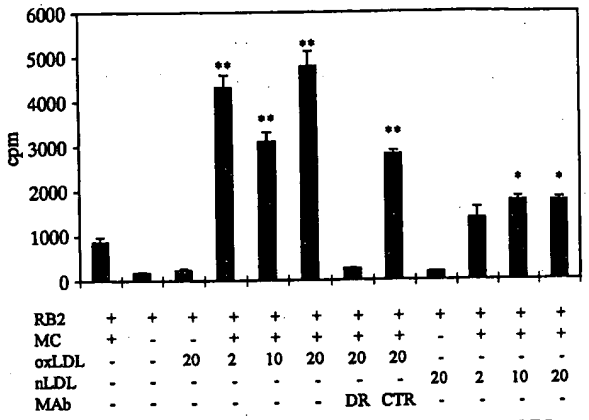

FIG. 1. Plaque T-cell clone RB2 was activated by oxLDL at concentrations of 2–20 μg/ml but not by nLDL. Activation was dependent on the presence of autologous PBMC (MC) and inhibited by the anti-HLA-DR monoclonal antibody L243 (DR) but not by the control monoclonal antibody MOPC 21 (CTR). Activation was determined as incorporation of [^{3}H]thymidine into DNA and is presented as cpm per well (mean ± SEM) of duplicate cultures. An asterisk indicates a statistically significant difference from control (left bar) by the Mann–Whitney U test (*, $P < 0.05$; **, $P < 0.01$). MAb, monoclonal antibody.

The monocyte-dependent oxLDL response of RB2 could be blocked with the monoclonal antibody L243, which reacts with HLA-DR, but not with the control monoclonal MOPC 21 (Fig. 1). This strongly suggests that the response to oxLDL represented an immunospecific T-cell activation by an HLA class II-restricted antigen presented by autologous monocytes.

No distinctive dose–response relationship could be discerned in the pattern of reactivity of this clone to oxLDL at 2–20 μg/ml (Fig. 1), and the response was of a significantly lower magnitude than that mounted when antigen receptor complexes were cross-linked on the surface of RB2 by the anti-CD3 antibody OKT3 (data not shown). This might be partly explained by a growth-inhibitory effect of oxLDL at higher concentrations. However, no cytotoxic effects of oxLDL were observed in our experiments (see Fig. 4). A low-level proliferative response could be seen when nLDL was used as an antigen instead of oxLDL (Fig. 1). This might be due to the minimal oxidation of the LDL preparation that occurs despite precautions (11).

HLA-DR-restricted oxLDL activation was observed with several other plaque CD4$^+$ clones from the three libraries. The responses of clones NN13, KB12, and RB16 are displayed in Fig. 2. As can be seen, T-cell proliferation of these clones was also stimulated by oxLDL at 2–20 μg/ml. Again, the responses were inhibited by anti-HLA-DR but not by MOPC 21, and they were dependent on the presence of autologous monocytes in the coculture system (Fig. 2).

A summary of the proliferative responses of all CD4$^+$ clones to oxLDL is displayed in Fig. 3. In total, six different preparations of LDL were tested for activation of T-cell clones. Four of 27 tested plaque-derived clones responded with a stimulation index between 3 and 5. These clones were immunospecific for oxLDL when applying the criteria of a >3-fold increase in DNA synthesis, dependence on autologous monocytes, and inhibition by anti-HLA-DR antibodies. None of the peripheral blood CD4$^+$ clones from any of the three patients exhibited significant responses to oxLDL (Fig. 3). Interestingly, two of the plaque-derived clones showed a modest response—i.e., stimulation indices between 2 and 3—with nLDL (Figs. 1 and 3). This suggests that these clones also react with LDL that has been modified by the cells in the coculture system.

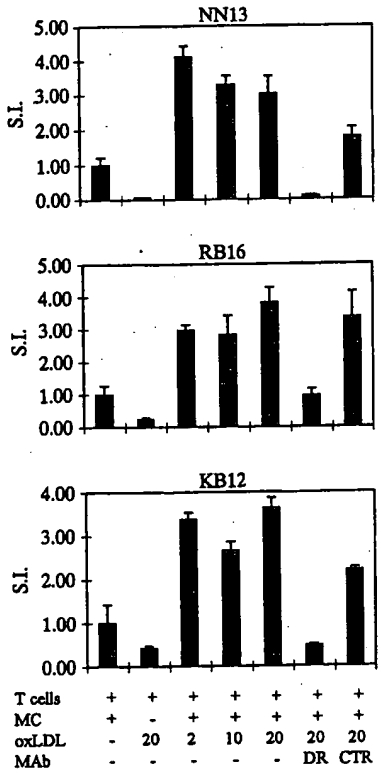

FIG. 2. Activation of three different plaque T-cell clones, NN13, RB16, and KB12, by oxLDL in the presence of autologous PBMC (MC). The response was inhibited by anti-HLA-DR (DR) but not by MOPC 21 (CTR). Data are presented as stimulation index [S.I., defined as the cpm of the actual sample divided by the cpm of the T cell/PBMC coculture in the absence of oxLDL (lane 1)]. Values shown are means ± SEM of duplicate cultures. MAb, monoclonal antibody.

To exclude the possibility that the observed proliferative response in the presence of oxLDL was due to a nonspecific enhancement by oxLDL of the immune recognition of non-LDL antigens present in the cultures, the effect of oxLDL on the antigen recognition by tetanus toxoid-specific T-cell clones was tested. Tetanus toxoid-specific T-cell clones were generated from the peripheral blood of a healthy donor. Fig. 4 shows that this clone responds to tetanus toxoid but not to oxLDL and that the rate of proliferation in response to tetanus toxoid is not affected by the simultaneous presence of oxLDL or nLDL. Two more tetanus toxoid-specific T cell clones were tested with identical results (data not shown). These results demonstrate that neither oxLDL nor nLDL enhances non-LDL-related immune recognition and support the conclusion that the oxLDL-dependent proliferative response of the plaque-derived T-cell clones is due to an immunospecific recognition of oxLDL.

The oxLDL-reactive clones secreted IFN-γ upon maximal stimulation with a CD3-crosslinking antibody (stimulation index, 629 ± 300; mean ± SD). In contrast, only one of the clones (RB16) produced significant amounts of IL-4 under the same conditions (stimulation index, 36). The responses to oxLDL stimulation under antigen-presenting conditions were generally of a lower magnitude than those exhibited towards anti-CD3 stimulation (data not shown).

DISCUSSION

The results of the present study demonstrate that human atherosclerotic plaques contain CD4$^+$ T cells immunospecific

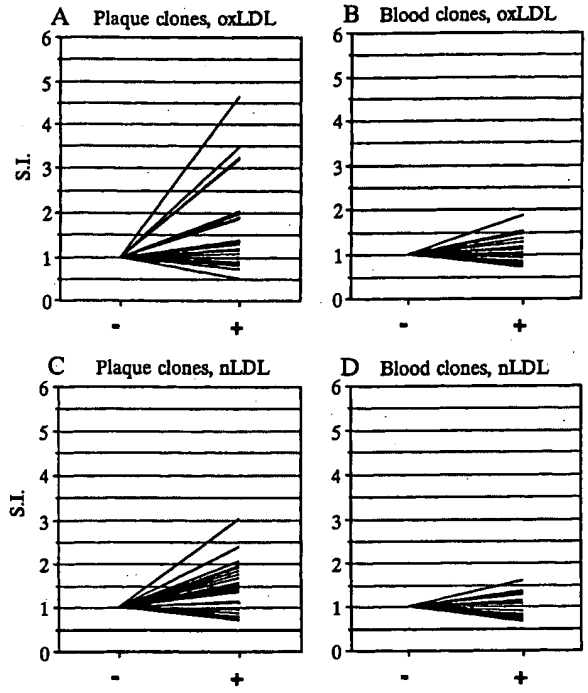

FIG. 3. Summary of LDL-induced activation of T-cell clones isolated from either plaques or peripheral blood. (*A*) Stimulation of T-cell clones derived from plaques with oxLDL. (*B*) Stimulation of T-cell clones derived from peripheral blood with oxLDL. (*C*) Stimulation of plaque T-cell clones with nLDL. (*D*) Stimulation of blood T-cell clones with nLDL. +, Incubation of T-cell clones with oxLDL or nLDL, respectively, in the presence of antigen-presenting PBMC. −, Incubation of T-cell clones with PBMC in the absence of oxLDL or nLDL. S.I., Stimulation index, as explained in the legend to Fig. 2.

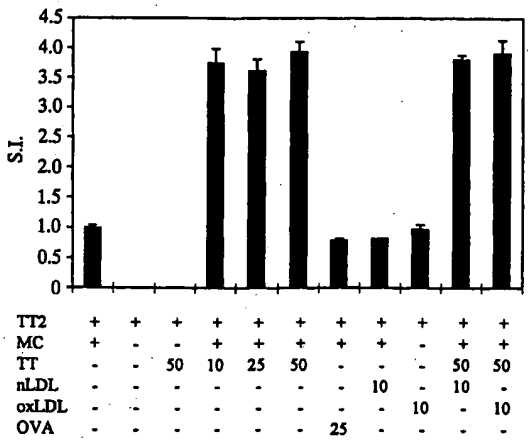

FIG. 4. Test of the effects of oxLDL and nLDL on the proliferative response to tetanus toxoid (TT) by a tetanus toxoid-specific T-cell clone TT2. TT2 cells were activated in the presence of autologous PBMC (MC) by tetanus toxoid but not by ovalbumin (OVA), oxLDL, or nLDL. The rate of proliferation in response to tetanus toxoid was not affected by the simultaneous presence of either oxLDL or nLDL. Figures below bars indicate concentrations in μg/ml. S.I., stimulation index, as explained in the legend to Fig. 2. Values shown are means ± SEM of duplicate cultures.

for oxLDL. This report, to the best of our knowledge, is the first identification of an immunologic specificity of T lymphocytes in human atherosclerotic plaques. Our conclusion is based on the responses of three clonal libraries established from advanced atherosclerotic plaques. At least one oxLDL-reactive clone was identified among the 8–10 clones tested in each library. In contrast, no oxLDL-specific clones were identified in libraries established from the peripheral blood of the same patients. However, no certain conclusions regarding the frequency of oxLDL-specific T cells in the atherosclerotic plaque could be made by using the current approach. Instead, such data await limiting dilution assays. The clones described here should, however, be useful for characterizing the specific epitopes, receptors, and restriction elements involved in the T-cell response to oxLDL.

The response of the oxLDL-specific $CD4^+$ T cells was dependent on the presence of autologous, antigen-presenting monocytes and was blocked by antibodies to HLA-DR. This strongly suggests that the T-cell response was mounted against an HLA-DR-restricted, processed antigen derived from oxLDL.

LDL is a heterogeneous particle that contains apolipoprotein B-100, cholesteryl esters, cholesterol, triacylglycerol, and phospholipids. Oxidative modification of the lipid moieties includes degradation of polyunsaturated fatty acid residues and generation of highly reactive aldehydes and other products that can modify other lipids. The cholesterol moiety also undergoes oxidation, and apolipoprotein B undergoes nonenzymatic degradation, as well as derivatization of its lysine and other residues (32–35). Several of these modifications represent neoantigens that may elicit antibody production (11). For example, both 4-hydroxynonenal- and malondialdehyde-modified lysine act as B-cell epitopes and are present in atherosclerotic plaques (8–11). Because oxidation of LDL represents a complex and heterogeneous series of reactions, it is likely that many structures are formed that may serve as neoantigens and give rise to many different T- and B-cell clones reactive against a large variety of epitopes.

The T-cell epitopes generated during oxidation of LDL which were recognized by these clones are at present unknown. The data of the present report allow a few predictions to be made. First, the response of the T-cell clones required the presence of antigen-presenting PBMC. This suggests that lipoprotein oxidation must be followed by receptor-mediated uptake and intracellular, lysosomal degradation to generate the mature T-cell epitopes. It is also likely that intracellular processing is necessary for the association of the T-cell epitopes of oxLDL to HLA-DR. Second, the low-magnitude, clonal T-cell response to nLDL in the presence of monocytes suggests that the latter cells generated the relevant epitopes during the coculture period—e.g., by release of oxygen radicals that generated a low level of oxidation of LDL. T-cell epitopes were therefore probably generated both by copper oxidation and by monocyte-dependent oxidation, and because the response was clonal, it is likely that the same epitopes were generated in both reactions. The rather modest degree of oxidation involved (≈10–20 nmol of thiobarbituric acid reactive substances per mg of protein) suggests that the T-cell epitopes recognized in our system were generated relatively early during the oxidative modification of LDL. It will now be important to identify the individual epitopes involved and to determine whether they are native or modified peptides from the apoprotein component of LDL.

LDL is present in high concentration in atherosclerotic plaques, where oxidation may occur via several mechanisms (8, 32). Presumably, this generates large amounts of antigen during the progression of the disease (2). As mentioned, antibody responses are mounted against oxLDL (9, 11). Very few B cells are, however, present in the plaque (20). In contrast, T cells are quantitatively important cellular components of the plaque throughout its development (20). Most of these T cells express the $CD45RO^+$ phenotype (14), which is expressed by T cells that, after their first antigen encounter in

lymph nodes, migrate to peripheral tissues (36). There, they can respond to antigenic challenge and perform effector functions. The observation that oxLDL stimulates DNA synthesis in bulk cultures of peripheral-blood T cells (37) might be explained by the presence in the blood of oxLDL-reactive memory T cells en route to peripheral tissues.

The secretory pattern of $CD4^+$ cells upon activation can be functionally divided into the T_H1 and T_H2 types, although the basis for these categories in man may not be as clearcut as it is in mice (38). In the case of oxLDL-responding T cells in the plaque, our data suggest that secretion of IFN-γ is more important than IL-4 secretion. The latter induces B-cell differentiation and antibody production, whereas the former activates macrophages, resulting in inflammatory responses (38). This would be in line with data showing few B cells but large amounts of activated macrophages (3, 17, 20, 22) and secretion of both monokines (39, 40) and IFN-γ (16) in plaques. The B-cell stimulation elicited by an initial T-cell response to oxLDL may, however, not be negligible, since antibodies to oxLDL are produced in high titers in apolipoprotein E knock-out mice that develop severe atherosclerosis but not in closely related mice that do not have atherosclerosis (41). Similarly, a high titer of such antibodies predict progression of carotid atherosclerosis in humans (12).

An autoimmune response to oxLDL may be of significant pathogenetic importance in atherosclerosis. Induction of antibody production would promote the elimination of oxLDL via Fc receptors (42). Secretion of IFN-γ would be expected to promote macrophage activation (43) and down-regulate scavenger receptor expression and intracellular cholesterol accumulation (44, 45). It also inhibits smooth muscle proliferation (46), α-actin expression (47), and collagen formation (48). Together with tumor necrosis factor, IFN-γ induces production of the vessel-relaxing factor, nitric oxide (49). Several of these phenomena have been demonstrated to occur *in vivo* and are likely to inhibit the fibrotic, stenosing process. This could be beneficial for the affected individual. It is, however, also possible that macrophage activation and the loss of structural proteins could increase the risk for plaque rupture and the formation of aneurysms. In conclusion, therefore, it is reasonable to suggest that the cellular immune response in the atherosclerotic plaque plays a modulating role in the development of the disease. Further experiments will be necessary to determine whether it is essentially beneficial or destructive.

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HUMAN PHYSIOLOGY

The Mechanisms of Body Function

Fourth Edition

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DEFENSE MECHANISMS OF THE BODY: IMMUNOLOGY, FOREIGN CHEMICALS, AND STRESS

17

SECTION A.
IMMUNOLOGY: THE BODY'S DEFENSES AGAINST FOREIGN MATTER

Immunity constitutes all the physiological mechanisms which allow the body to recognize materials as foreign or abnormal and to neutralize or eliminate them; in essence, these mechanisms maintain uniqueness of "self." Classically, immunity referred to the resistance of the body to **microbes** (viruses, bacteria, and protozoa), as well as to fungi and multicellular organisms such as parasitic worms. It is now recognized, however, that the immune system has more diverse functions than this. It is involved in the elimination of "worn-out" or damaged body cells (such as old erythrocytes) and in the destruction of abnormal or mutant cells which arise within the body. This last function, known as **immune surveillance**, constitutes a major defense against cancer.

It has also become evident that immune responses are not always beneficial and may result in serious damage to the body. In addition, the immune system seems to be involved in the process of aging. Finally, it constitutes the major obstacle to successful transplantation of organs. Because of these broad relationships, few other research areas of biology have grown so rapidly or have produced such a wealth of new and exciting, albeit sometimes bewildering, information.

Immune responses may be classified into two categories: specific and nonspecific. **Specific immune responses**, which are mediated by lymphocytes (and cells derived from lymphocytes), depend upon prior exposure to a specific foreign material, recognition of it upon subsequent exposure, and reaction to it. In contrast, the **nonspecific immune responses** do not require previous exposure to the particular foreign material, and they nonselectively protect against foreign materials without having to recognize their specific identities. The nonspecific immune responses are particularly important during the initial exposure to a foreign organism.

Immune responses can be viewed in the same way as other homeostatic processes in the body, i.e., as stimulus-response sequences of events. In such an analysis, the groups of cells which mediate the final responses are effector cells. Before we begin our description of these effector cells, let us first at least mention some of their major opponents—bacteria and viruses.

Bacteria are unicellular organisms which have not only a cell membrane but also an outer coating, the cell wall. Most bacteria are self-contained complete cells in that they have all the machinery required to sustain life and reproduce themselves. In contrast, **viruses** are essentially nucleic acids that are surrounded by a protein coat. They lack both the enzyme machinery for energy production and the ribosomes essential for protein synthesis. Thus, they cannot survive by themselves but must "live" inside other cells whose biochemical apparatus they make use of. The viral nucleic acid directs the synthesis by the host cell of the proteins required for viral replication, with nucleotides and energy sources also being supplied by the host cell. Other types of microorganisms and multicellular parasites are potentially harmful to human beings, but we shall devote most of our attention to the body's defense mechanisms against bacteria and viruses.

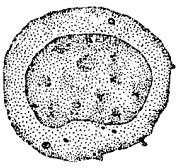
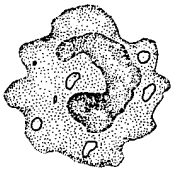
How microorganisms cause damage and endanger health depends upon the specific bacterium or virus involved. Some bacteria directly destroy cells by releasing enzymes which break down nearby cell membranes and organelles. Others give off toxins which disrupt the functions of organs and tissues. The effect of viral habitation and replication within a cell depends upon the type of virus. Some viral particles, after entering a cell, multiply very rapidly and kill the cell by depleting it of essential components or by directing it to produce toxic substances; with death of the host cell, the viral particles leave and move on to another cell. In contrast, other viral particles replicate inside their host cells very slowly, and the viral nucleic acid may even become associated with the cell's own DNA molecules, replicating along with them and being passed on to the daughter cells during cell division;

such a virus may remain in the cell or its offspring for many years. As we shall see, cells infected with these so-called **slow viruses** may be damaged by the body's own defense mechanisms turned against the cells because they are no longer "recognized" as "self." Finally, it is likely that certain viruses cause transformation of their host cells into cancer cells.

Effector Cells of the Immune System

The major types of effector cells of the immune system (Table 17-1) are the different types of leukocytes (white blood cells), the plasma cells, and the macrophages. (All the functions listed in Table 17-1 will be described in subsequent sections and are presented here together for orientation and reference.) The **leukocyte** types (**neutrophils, eosinophils, basophils, monocytes,** and **lymphocytes**) were described in Chap. 11 and should be reviewed at this time. **Plasma cells** are derived from lymphocytes and are the cells which secrete antibodies. **Macrophages** have multiple functions, including phagocytosis, and are found scattered throughout the tissues of the body (the structure of these cells varies from place to place). Some macrophages are capable of replication, but the major source of tis-

TABLE 17-1. Major effector-cell types of the immune system

	White blood cells (leukocytes)						
	Polymorphonuclear granulocytes						
	Neutrophils	Eosinophils	Basophils	Lymphocytes	Monocytes	Plasma cells	Macrophages
							
Percent of total leukocytes	50–70	1–4	0.1	20–40	2–8		
Primary sites of production	Bone marrow	Bone marrow	Bone marrow	Bone marrow, thymus, and lymphoid tissues	Bone marrow	Derived from B lymphocytes in lymphoid tissue	Most are formed from monocytes
Primary known function	Phagocytosis; release of chemicals involved in inflammation (chemotaxins, etc.)	Destruction of parasitic worms	Release of histamine and other chemicals; transformed into mast cells with similar functions	B cells: production of antibodies (after transformation into plasma cells) T cells: different subgroups responsible for cell-mediated immunity, "helping" or suppressing B cells and other T cells	Transformed into tissue macrophages; functions similar to macrophages	Production of antibodies	Phagocytosis; assist in antibody formation and T-cell sensitization; secretion of chemicals involved in inflammation, regulation of lymphocytes, and total-body response to infection or injury

sue macrophages is by influx of blood monocytes and their differentiation locally into macrophages. Monocytes and macrophages are often termed the **mononuclear phagocyte system** to distinguish them from the other major phagocytes of the body, the neutrophils (eosinophils are also phagocytic, but as shown in Table 17-1, they are few in number and are highly selective in their targets). Because of the large number of cell types and chemical mediators involved in immune responses, a miniglossary defining them is given at the end of the section on immunology.

The effector cells of the immune system are distributed throughout the organs and tissues of the body, but are found particularly in the so-called **lymphoid tissues** (Fig. 17-1 and Table 17-2)—lymph nodes, spleen, thymus, aggregates of lymphoid follicles associated with the gastrointestinal tract (for example, the tonsils), and bone marrow.

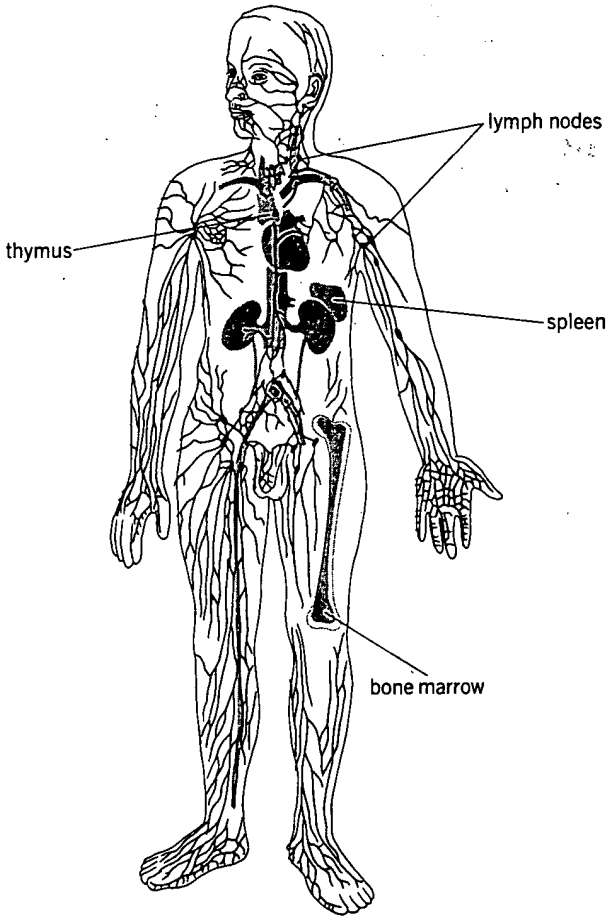


FIGURE 17-1. Location of various lymphoid organs: lymph nodes, thymus, and spleen. Not shown are the tonsils and other lymphoid patches in the various epithelial linings of the body. The bone marrow is the site of production of many of the cells which come to reside in the other lymphoid organs.

Lymph nodes function as filters along the course of the lymph vessels, lymph flowing through them before being returned to the general circulation. Lymph enters the node via afferent lymphatic vessels, trickles through the lymphatic sinuses of the node, and leaves via the efferent lymphatic vessels on the other side. The lymphatic sinuses are relatively open channels, and as the lymph flows through them, some lymphocytes are removed to be stored temporarily in the node and others are added to the lymph. Some of the lymphocytes released into the lymph are those previously stored, but others have been newly formed, after an appropriate stimulus, in the node. The lymphatic sinuses are lined with macrophages, which phagocytize particulate matter, such as dust (inhaled into the lungs), cellular debris, bacteria, and other microorganisms. Certain lymphocytes in the lymph nodes can differentiate into plasma cells which secrete antibodies.

The **spleen** is the largest of the lymphoid organs and lies in the left part of the abdominal cavity between the stomach and the diaphragm. The interior of the spleen is filled with a reticular meshwork, the **red pulp** and the **white pulp**. Blood, rather than lymph, percolates through the red pulp (thus its name), and erythrocytes as well as lymphocytes and macrophages are collected in the spaces in the meshwork. In the human fetus, the spleen is an important blood-cell-forming organ, but in the adult only lymphocytes are formed there. They are produced in the white pulp of the spleen. The macrophages of the spleen phagocytize many of the products of red cell degradation as well as various kinds of foreign matter. In essence, the spleen is to the circulating blood what the lymph nodes are to the lymph. Plasma cells derived from lymphocytes in the spleen, as in the lymph nodes, produce antibodies.

The **thymus** lies in the upper part of the chest, and its size varies with age; it is relatively large at birth and grows until puberty (10 to 15 years of age)

TABLE 17-2. Some functions of lymphoid organs

Lymph nodes (including unencapsulated lymphoid tissues)	1. Produce antibodies and sensitized T cells 2. Remove and store lymphocytes 3. Form and add new lymphocytes to lymph flow through nodes 4. Remove particulate matter through phagocytosis by macrophages
Spleen	1. Produce antibodies and sensitized T cells 2. Produce red blood cells in fetus but not adult 3. Produce new lymphocytes 4. Remove products of red-cell degradation and other foreign matter through phagocytosis by macrophages 5. Store red blood cells, which can be added to circulation by contraction of the spleen
Thymus	1. Produce T lymphocytes 2. Secrete hormones (thymosin)

when it gradually atrophies and is replaced by fatty tissue. The thymus is the organ responsible for differentiation of one of the major classes of lymphocyte (T lymphocytes); it also secretes a group of hormones known collectively as **thymosin**.

The **bone marrow** is also classified as a lymphoid organ because it produces lymphocytes. Indeed, the bone marrow is the source of virtually all the lymphocytes which "seed" the other lymphoid tissues (this will be described more fully in a subsequent section); thus the ancestry of almost all the lymphocytes in an adult can be traced back to the bone marrow [in fact, this is true of all effector cells of the immune response (Fig. 17-2)]. However, unlike the lymph nodes and spleen, the bone marrow is not a site in which lymphocytes become activated to secrete antibodies or participate in other specific immune responses.

Nonspecific Immune Responses

External anatomical and chemical "barriers"

The body's first lines of defense against infection are the barriers offered by surfaces exposed to the external environment. Very few microorganisms can penetrate the intact skin, and the sweat, sebaceous, and lacrimal glands secrete chemical substances which are highly toxic to certain forms of bacteria. The mucous membranes also contain antimicrobial chemicals, but more important, mucus is sticky. When particles adhere to it, they can be swept away by ciliary action, as occurs in the upper respiratory tract, or engulfed by phagocytic cells. Other specialized surface "barriers" are the hairs at the entrance to the nose, the cough reflexes, and the acid secretion of the stomach. Finally, a major "barrier" to infection is the normal microbial flora of the skin and other linings exposed to the external environment; these microbes suppress the growth of other potentially more virulent ones.

Inflammatory response

Despite the effectiveness of the external barriers, small numbers of microorganisms penetrate them every day. Think of all the small breaks produced in the skin or mucous membranes by tooth-brushing, shaving, tiny scratches, etc. In addition, we now recognize that many viruses are able to penetrate seemingly intact healthy skin or mucous membranes.

Once the invader has gained entry, it triggers off **inflammation**, the response to injury. The local

manifestations of the inflammatory response are a complex sequence of highly interrelated events, the overall functions of which are to bring phagocytes into the damaged area so that they can destroy (or inactivate) the foreign invaders and set the stage for tissue repair. The sequence of events which constitute the inflammatory response varies, depending upon the injurious agent (bacteria, cold, heat, trauma, etc.), the site of injury, and the state of the body, but the similarities are in many respects more striking than the differences. It should be emphasized that, in this section, we describe inflammation in its most basic form, i.e., the *nonspecific* innate response to foreign material. As we shall see, inflammation remains the basic scenario for the acting out of *specific* immune responses as well, the difference being that the entire process is amplified and made more efficient by the participation of antibodies and sensitized lymphocytes, i.e., by agents of specific immune responses.

The sequence of events in an inflammatory response is briefly as follows, using bacterial infection as our example:

1. Initial entry of bacteria
2. Vasodilation of the vessels of the microcirculation leading to increased blood flow
3. Marked increase in vascular permeability to protein
4. Filtration of fluid into the tissue with resultant swelling
5. Exit of neutrophils (and, later, monocytes) from the vessels into the tissues
6. Destruction of the bacteria either through phagocytosis and intracellular killing, or by mechanisms not requiring prior phagocytosis
7. Tissue repair

The familiar gross manifestations of this process are redness, swelling, heat, and pain, the latter being the result both of distension and of the effect of locally produced substances on afferent nerve endings.

Each of the events of inflammation is induced and regulated by chemical mediators of varying origins. Surprisingly, some are produced by the bacteria themselves. Some are released from tissue cells present in the area prior to the invasion; an example is **histamine**, present in mast cells scattered throughout the tissues of the body and a potent inducer of steps 2 to 4. Other chemical mediators are secreted by neutrophils and mononuclear phagocytes which enter the area (step 5). Still others are newly generated in the interstitial fluid of the area from inactive plasma precursors; an example is the **kinins**, peptides which are split in the inflamed area from proteins (**kininogens**) circulating in the blood (Chap. 11), and which not only stimulate steps 2 to 5 but may activate neuronal pain receptors.

There are a very large number of these chemical mediators and an even larger number of stimuli for their release from cells or generation in the interstitial fluid. Therefore, we shall limit our naming of them to a few examples (like histamine and the kinins) and the single most important group of mediators, known as **complement**, which will be discussed later.

Vasodilation and increased permeability to protein. Immediately upon microbial entry, chemical mediators dilate most of the vessels of the microcirculation in the area and somehow alter the material between the endothelial cells so as to make the capillaries quite leaky to large molecules. Tissue swelling is directly related to these changes: The arteriolar dilation increases capillary blood pressure, thereby favoring filtration of fluid out of the capillaries; more important, the protein which leaks out of the vessels as a result of increased permeability builds up locally in the interstitium, thereby diminishing the difference in protein concentration between plasma and interstitium (recall from Chap. 11 that this difference is mainly responsible for the osmotic flow of fluid from interstitium into capillaries).

The adaptive value of all these vascular changes is twofold: (1) The increased blood flow to the inflamed area increases the delivery of phagocytic leukocytes and plasma proteins crucial for immune responses; and (2) the increased capillary permeability to protein ensures that the relevant plasma proteins—all normally restrained by the

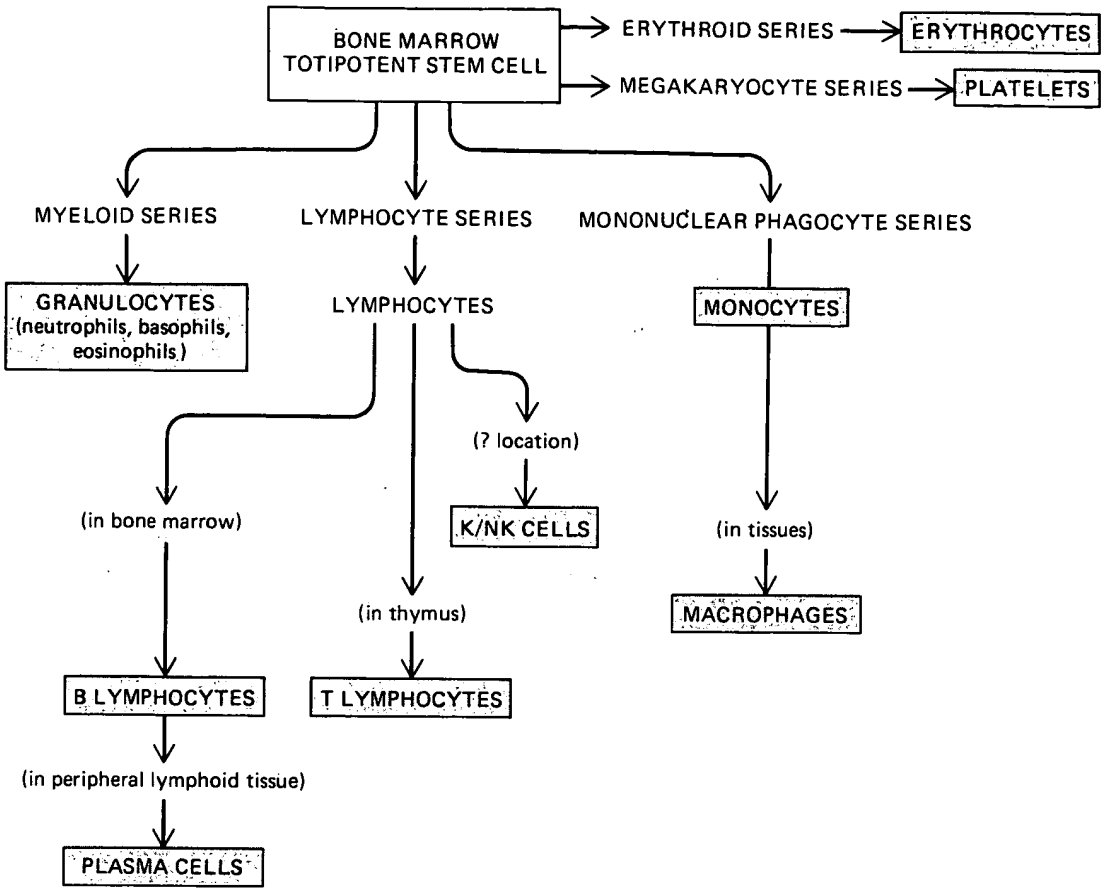


FIGURE 17-2. Origins of effector cells of immune responses. The bone marrow produces granulocytes, lymphocytes, and monocytes (as well as erythrocytes and platelets). Further transformation of the monocytes (into macrophages), B lymphocytes (into plasma cells), and lymphocytes destined to be T lymphocytes occurs in sites outside the bone marrow. K/NK cells are described later.

capillary membranes—can gain entry to the inflamed area.

Chemotaxis. Within 30 to 60 min after the onset of inflammation, a remarkable interaction occurs between the capillary endothelium and circulating neutrophils. First, the blood-borne neutrophils begin to stick to the inner surface of the endothelium. Following their surface attachment, the neutrophils manifest considerable amoebalike activity. Soon a narrow amoeboid projection is inserted into the space between two endothelial cells and the entire neutrophil then squeezes into the interstitium (**neutrophil exudation**). The neutrophil may simply pry the intercellular connections apart by the force of its amoeboid movement, or it may secrete substances which chemically disrupt the connection. In this way, huge numbers of neutrophils migrate into the inflamed areas of tissue and move toward the microbes. This entire response of the neutrophils is known as **chemotaxis** and is induced by chemical mediators (**chemotaxins**) which move out from the inflamed area by diffusion, forming a chemotaxin concentration gradient, with the area immediately around the microbes containing the greatest concentration. This permits the chemotaxins to polarize the motion of the neutrophils along this gradient. The neutrophils do not swim but

rather "crawl" toward the microbe. The process is initiated by the binding of the chemotaxins to plasma-membrane receptors on the neutrophil's surface, and this binding activates increased entry of calcium into the neutrophil's cytosol; it is this calcium which activates the force-generating processes associated with actin microfilaments that lead to amoeboid motion along the chemotaxin gradient.

Movement of leukocytes into the tissue is not limited only to neutrophils. Monocytes follow, but usually later, and once in the tissue are transformed into macrophages. Meanwhile some of the macrophages normally present in the tissue may multiply by mitosis and become motile. Thus, all the major phagocytic cell types are present in the inflamed area. Usually the neutrophils predominate early in the infection but tend to die off more rapidly than the others, thereby yielding a more mononuclear picture later. In contrast, in certain types of allergies and inflammatory responses to parasites, eosinophils are in striking preponderance.

Phagocytosis. Phagocytosis is a primary function of the inflammatory response, and the increased blood flow, vascular permeability, and leukocyte exudation mentioned above serve largely to ensure the presence of adequate numbers of phagocytes and to provide the milieu required for the performance of their function.

The initial step in phagocytosis is contact between the surfaces of the phagocyte and microbe (or foreign particle or damaged native cell). But such contact is not, itself, always sufficient to cause firm attachment and trigger engulfment. This is particularly true for many virulent bacteria which have a thick polysaccharide capsule that makes attachment and engulfment very difficult. As we shall see, the presence of molecular factors (produced by the body), which actually bind the phagocyte to the object it is to engulf, markedly enhances the process of phagocytosis. (Any substance that does this is known as an **opsonin**, from the Greek word that means "to prepare for eating.")

The process of ingestion is illustrated in Fig. 17-3. The phagocyte engulfs the organism by endocytosis, i.e., membrane invagination and pouch (**phagosome**) formation. Once inside, the microbe remains in the phagosome, a layer of plasma membrane separating it from the phagocyte's cytosol. The next step is known as **degranulation** (Fig. 17-3): The membrane surrounding the phagosome makes contact with one of the phagocyte's lysosomes (these appear microscopically as "granules") which are filled with a variety of hydrolytic enzymes. Once contact between the phagosome and lysosome is made, the membranes fuse (the disappearance of the granules during this fusion leads to the term "degranulation"), and the combined vesicles are now called the **phagolysosome**,

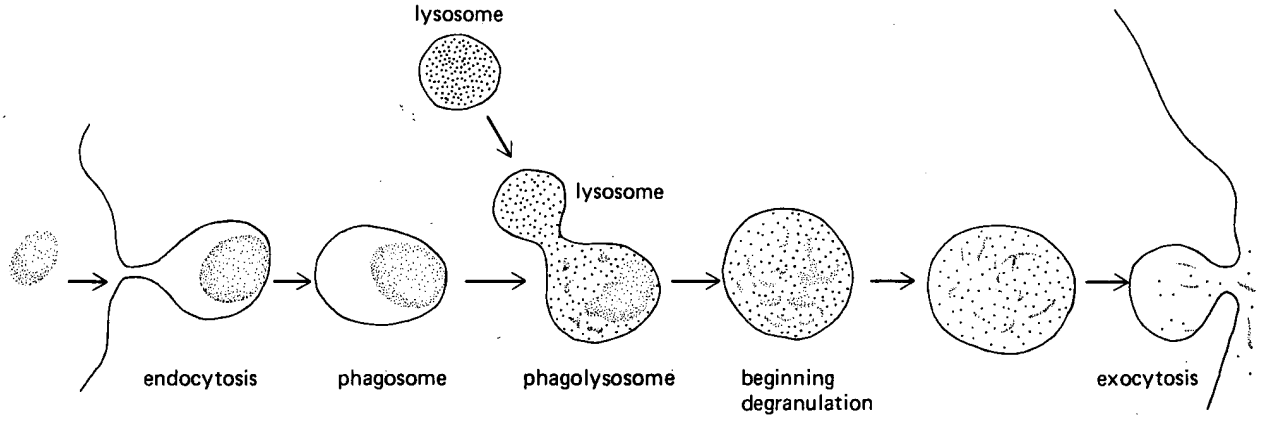


FIGURE 17-3. Large molecules and particulate substances enter the cell by endocytosis, forming a membrane-bound phagosome (left). A phagosome may then merge with a lysosome, which brings together the digestive enzymes of the lysosome and the contents of the phagosome. After digestion has taken place, the contents may be released to the outside of the cell by exocytosis.

inside which the microbe is exposed to the lysosomal enzymes capable of breaking down its macromolecules.

However, the lysosomal enzymes are not the major mechanisms within the phagolysosome for killing bacteria. The phagolysosome produces a high concentration of hydrogen peroxide and other derivatives of oxygen, which are extremely destructive to macromolecules. This production is mediated by enzymes in the phagolysosomal membrane, activated in response to the presence of the foreign particle.

The phagolysosomal enzymes and oxygen derivatives not only kill the microorganism but catabolize it into low-molecular-weight products which can then be safely released from the phagocyte or actually utilized by the cell in its own metabolic processes. This entire process need not kill the phagocyte, which may repeat its function over and over before dying. Nondegradable foreign particles (such as wood, tattoo dyes, or metal) and certain species of microorganisms may be retained indefinitely within macrophages.

Neutrophils and macrophages function in the inflammatory process not only as phagocytes but as secretory cells (Fig. 17-4). Contact with microbes stimulates them to release one type of their lysosomes not into the phagosome but into the extracellular fluid. In addition to releasing these preformed intravesicle chemicals, they are also stimulated to synthesize and release many other substances. In the extracellular fluid, some of these chemicals (both those newly synthesized and those released from vesicles) function as mediators of the inflammatory response. For example, several chemicals secreted by the neutrophil induce histamine release from nearby mast cells and generate kinins from kininogens in the extracellular fluid.

Some of the other substances released by the phagocyte into the extracellular fluid are toxic to microbes (as will be described in the next section). Still others are enzymes which can trigger both the clotting and anticlotting pathways described in Chap. 11. Still others, released specifically by macrophages (and their precursors, monocytes) enter the circulation and function as hormones to produce adaptive changes in the function of organs and tissues far removed from the inflammatory site (as will be described later).

Direct killing of microbes. Phagocytosis is not the only way of destroying microbes. As mentioned above, the phagocytes release into the extracellular fluid antimicrobial substances. For example, the enzymes which generate the antimicrobial oxygen derivatives within the phagolysosomes are also lo-

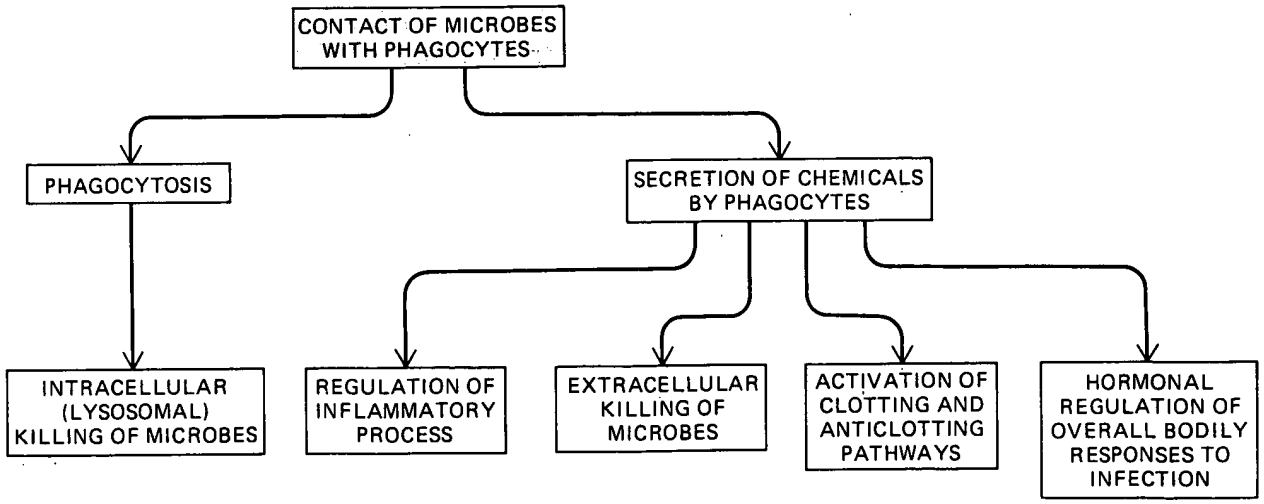


FIGURE 17-4. Role of phagocytes in nonspecific immune responses.

cated on the outer surface of the phagocyte's plasma membrane, and so these substances also gain entry to the extracellular fluid. So do certain digestive enzymes contained in the vesicles released extracellularly by the phagocyte. An example of a more subtle antimicrobial substance secreted by neutrophils into the extracellular fluid is **lactoferrin**, a protein which tightly binds iron and hence prevents the microbes, whose multiplication requires considerable iron, from obtaining it.

There are, in addition to the antimicrobial chemicals released into the extracellular fluid by phagocytes, at least two other mechanisms for destroying the cell without phagocytosis which act during the period prior to the generation of the specific immune responses. One of these involves a class of lymphocyte known as **natural killer (NK)** cells, but because its role is best established in the body's defenses against cancer and virus-infected cells, it will be described in those contexts. The remaining mechanism for early extracellular killing of microbes—the **complement** system—is the most important of all in nonspecific immune response against bacteria. However, its actions extend well beyond cell-killing, and so we will treat it in its entirety after describing the last stage in local inflammation—tissue repair.

Tissue repair. The final stage of the inflammatory process is tissue repair. Depending upon the tissue involved, regeneration of organ-specific cells may or may not occur (for example, regeneration occurs in skin and liver but not in the central nervous system). In addition, fibroblasts in the area divide rapidly and begin to secrete large quantities of collagen, forming **scar** tissue.

The end result may be complete repair (with or without a scar), **abscess** formation, or **granuloma** formation. An abscess is a bag of pus (microbes, leukocytes, and liquified debris) walled off by fibroblasts and collagen. This occurs when tissue breakdown is very severe and when the microbes cannot be eliminated but only contained. When this stage has been reached, the abscess must be drained, for it will not be absorbed spontaneously.

A granuloma occurs when the inflammation has been caused by certain microbes (such as the bacteria causing tuberculosis) which are engulfed by phagocytes but survive within them. It also occurs when the inflammatory agent is a nonmicrobial substance which cannot be digested by the phagocytes. The granuloma consists of numerous layers of phagocytic-type cells, the central ones of which contain the offending material. The whole thing is, itself, usually surrounded by a fibrous capsule. Thus, a person may harbor live tuberculosis-producing bacteria for many years and show no ill effects as long as the microbes are contained within a granuloma and are not allowed to escape.

The complement system. The **complement** system is yet another example (the clotting, anticlotting, and kinin systems are others) of a group of plasma proteins which normally circulate in the blood in an inactive state; upon activation of the first protein of the group, there occurs a sequential cascade in which active molecules are generated from inactive precursors. In the complement system, the final active protein generated in the cascade directly kills foreign cells by attacking their plasma membranes. This protein is really a complex of five different proteins known appropriately as the **membrane attack complex**, or **MAC**. MAC imbeds itself in the microbial surface and creates channels through the membrane, making it leaky and killing the microbe. This is the major mechanism whereby microbes can be killed directly without prior phagocytosis in nonspecific inflammatory responses.

Thus far, the complement system is analagous to the other cascade systems we have described, in that the final activated molecule (fibrin in clotting, plasmin in anticlotting, kinins in the kinin system) is the direct mediator of the biological response of that system. However, in the complement system, certain of the different active proteins generated along the cascade prior to the terminal protein also function as mediators of a particular inflammatory response (vasodilation, chemotaxis, etc.). Figure 17-5 illustrates these in terms of one of the most important complement molecules, C_3. (Since this system consists of at least 20 distinct proteins, it is extremely complex and we will make no attempt to

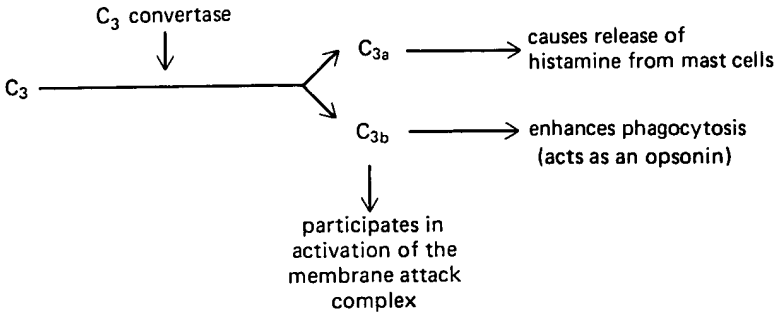


FIGURE 17-5. An example of events in the complement system. The complement protein C_3 is split into two proteins, C_{3a} and C_{3b}. C_{3a} functions as one of the mediators of the inflammatory response, in large part by stimulating mast cells to release histamine. C_{3b} attaches to the microbe where it not only facilitates phagocytosis but participates in the activation of the next complement protein in the sequence.

identify the roles of individual complement proteins except for purposes of illustration or clarity.)

As summarized in Fig. 17-6, one or more of the activated complement molecules is capable of mediating virtually every step of the inflammatory response. Certain of them enhance vasodilation and increased capillary permeability by stimulating release of histamine from mast cells and by generating kinins. Some stimulate neutrophil exudation by acting as powerful chemotaxic agents. Another complement component (C_{3b}) enhances phagocytosis, i.e., it acts as an opsonin (the only major one in nonspecific inflammatory responses), attaching the phagocyte to the object it is to engulf (Fig. 17-7). One portion of this complement molecule binds covalently and nonspecifically to the surface of the microbe, whereas another portion binds to specific receptor sites for it on the plasma membrane of the phagocyte. In this manner, the complement molecule forms a link between the two cells, providing the surface contact needed to initiate phagocytosis.

In describing the actions of the complement system we have emphasized the sequential or cascade nature of the reactions required for the generation of active mediators, but we have so far ignored the problem of just how the sequence is initiated. As we shall see in a later section, antibody is required to activate the very first protein (C_1) in the entire complement sequence now known as the **classical complement pathway**, but antibody is not present in the nonspecific inflammatory responses we are presently describing. There is, however, an **alternate pathway** for complement activation which is not antibody-dependent and which bypasses the

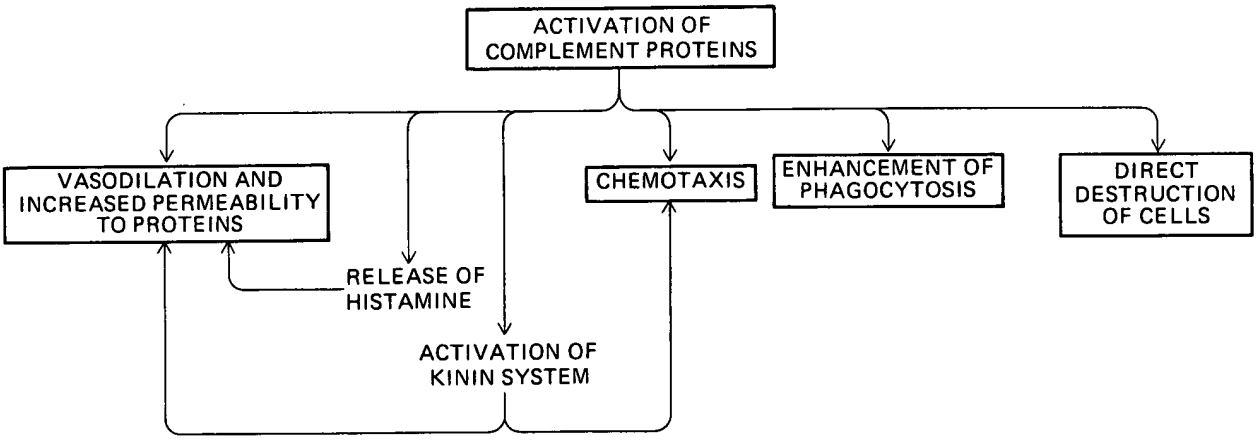


FIGURE 17-6. Functions of complement.

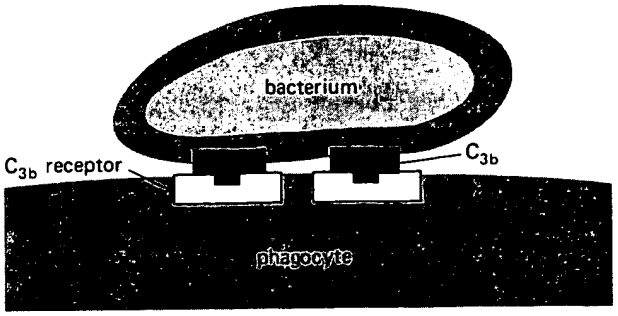


FIGURE 17-7. Function of complement C_{3b} as an opsonin. C_{3b} attaches to the bacterial surface by covalent bonds and to the phagocyte via plasma-membrane receptors for it. The structures in this figure are not drawn to scale.

first steps in the classical pathway. Relatively little biological activity is lost in bypassing the first steps, because no essential inflammatory mediators are generated by them. The first really critical step in this regard is the cleavage of C_3 to its subunits C_{3a} and C_{3b} (Fig. 17-5), and this is where the alternate pathway plugs in.

The critical question for initiation via the alternate pathway can, therefore, be stated as follows: How does the presence of microbes in the absence of antibodies lead to the generation of an enzyme (**C_3 convertase**) which can cleave C_3, and, thereby, initiate the rest of the complement pathway? Unfortunately, the answer to this question is still not completely clear, but appears to involve the participation of six alternate-pathway complement proteins. Suffice it to say that the the key element required to generate C_3 convertase from these proteins is the presence on the microbial surface of carbohydrate molecules not found on normal human cells. Moreover, as the C_{3b} proteins are formed by the action of C_3 convertase, they bind to the surface of the microbes, and this permits the remainder of the complement reactions, including the ultimate formation of the MAC, to proceed in the immediate vicinity of the microbe.

To recapitulate, the alternate pathway of complement activation provides a means of natural resistance to infectious agents and is brought into play even in the total absence of antibody. Thus, it provides an immediately available line of defense that does not require previous exposure to the invading microbe. It may also turn out that this alternate pathway plays an important role in attacking not only extracellular microbes but virus-infected host cells as well as cancer cells.

Systemic manifestations of inflammation

We have thus far described the *local* aspects of the inflammatory response. What are the *systemic* responses, i.e., responses of organs and tissues distant from the site of inflammation? Probably the single most common and striking sign of injury is fever. The substance primarily responsible for the resetting of the hypothalamic thermostat, as described in Chap. 15, is a protein known as **endogenous pyrogen**, which is secreted by monocytes and macrophages.

One would expect fever, being such a consistent concomitant of infection, to play some important protective role, and recent evidence strongly suggests that such is the case (this is a very important question in light of the widespread use of aspirin and other drugs to suppress fever). In contrast to the possible benefits of fever, there is no question that an extremely high fever may be quite harmful, particularly in its effects on the functioning of the central nervous system, and convulsions are not infrequent in young children with high fevers.

Another striking systemic change which occurs in response to infection or tissue injury is a decrease in the plasma concentrations of iron (and zinc), due to changes in the intake and/or release of iron by liver, spleen, and other tissues. This phenomenon is beneficial since, as mentioned before, bacteria require a high concentration of iron to multiply (recall that another way of reducing the iron available to microbes is release of an iron-binding protein by neutrophils in the inflamed area).

In addition, the liver is stimulated during infection to release a host of proteins known collectively as **acute phase proteins**, which exert a bewildering array of effects on the inflammatory process, immune cell function, and tissue repair. The bone marrow is stimulated to produce and release neutrophils and other granulocytes (**granulopoiesis**).

The eliciting of these last three effects (decreased plasma iron and zinc, release of acute phase proteins, and stimulation of granulopoiesis) is ascribed to a substance named **leukocyte endogenous mediator (LEM)** (Fig. 17-8), secreted by monocytes and macrophages. In fact, it is likely that LEM is the same substance as endogenous pyrogen, EP (or at least, a very close relative).

To add to the diversity of actions exerted by EP, it now appears that **interleukin 1 (IL-1)**, a protein which is secreted by macrophages and which is required for activation of lymphocytes, as we shall see, is also identical (or very similar) to EP. Thus, EP/LEM/IL-1 acts as hormone and paracrine to elicit a broad spectrum of events throughout the body which are adaptive in the response to infection or injury. (The list of effects given here is by no means complete.) Indeed, the release of EP/LEM/IL-1 is probably elicited by other stresses as part of a nonspecific protective response. For example, some of the physiological responses to endurance exercise closely parallel the response to infection, and EP release has been reported in this situation.

Interferon

Interferon, a family of proteins with differing amino acid sequences, sites of production, and ranges of biological activity is a major nonspecific defense mechanism, particularly against viral infection. Interferon nonspecifically inhibits viral replication inside host cells. In response to the presence of a virus (its nucleic acid is the inducer), interferon is synthesized and secreted into the extracellular fluid by many different cell types (including, but not limited to, some which are effector cells of the immune

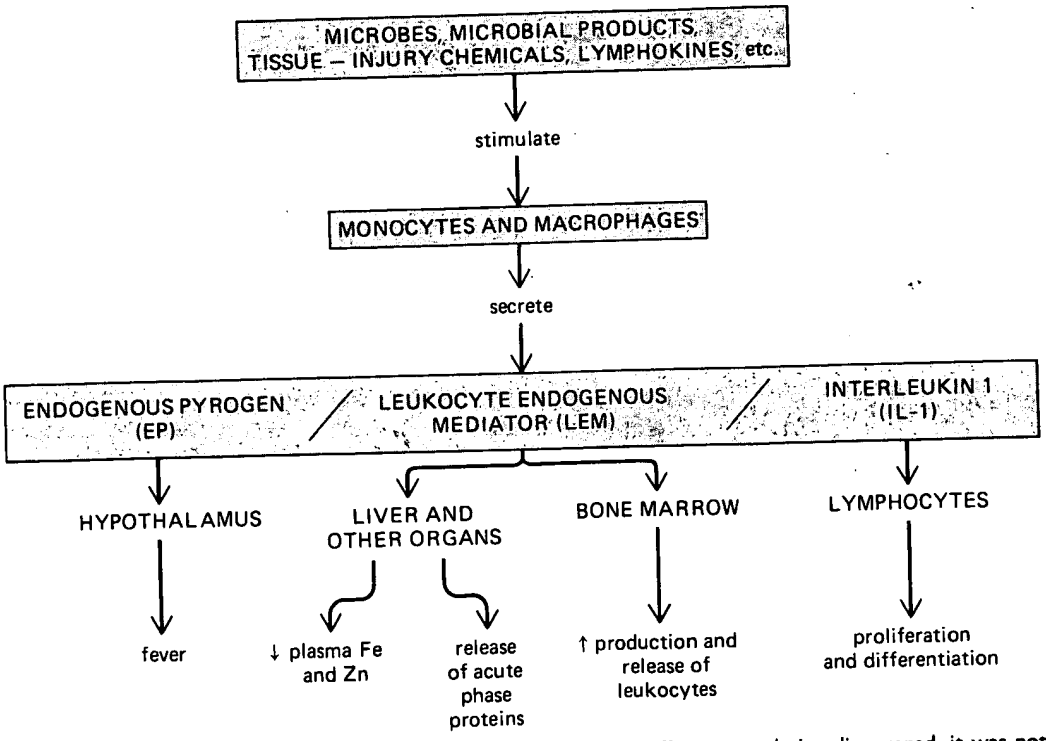


FIGURE 17-8. Origin and functions of EP/LEM/IL-1. When these effects were being discovered, it was not realized that they are probably due to the action of a single protein (or several very closely related proteins); rather, it was thought that three distinct proteins were involved (fever caused by EP, activation of the immune system by IL-1, etc.), and this accounts for the multiple names. *(Adapted from W. R. Beisel.)*

response, like macrophages and lymphocytes). It then binds to plasma membrane receptors on nearby bodily cells (additionally, some types of interferon may also enter the circulation and reach cells at far-removed sites).

The interferon molecule does not, itself, have direct antiviral activity; rather its binding to the plasma membrane triggers the synthesis of several enzymes by the cell to which it is bound, and these enzymes block synthesis within the cell of proteins the virus requires for its multiplication (Fig. 17-9). Importantly, the enzymes synthesized in response to interferon are inactive until the cell is actually infected by a virus (the virus's RNA conveniently serves as activator); this protects the cell's normal protein-synthesizing machinery from inhibition should the cell never be invaded. The fact that interferon itself is not antiviral but rather stimulates the cells to make their own antiviral proteins is another example of amplification—a single molecule of interferon can trigger the synthesis of many antiviral proteins so that only a few interferon molecules are required to protect a cell.

To reiterate, interferon is not specific; many viruses induce interferon synthesis, and interferon, in turn, can inhibit the multiplication of many different viruses. This nonspecificity permits the very rapidly reacting interferon system to contain a viral infection until the more slowly reacting specific immune responses can take over.

In this regard, one of the most important interactions between interferon and the specific immune system should be mentioned here (it will be described in more detail later). Interferon stimulates the activity of those lymphocytes which directly attack and kill virus-infected cells. Our description thus far has focused entirely on interferon's antiviral role. However, it is now known that other microbes, particularly those with an intracellular phase to their growth cycle, can induce cells to make interferon. It is likely that interferon helps to protect against these invaders as well. But the most

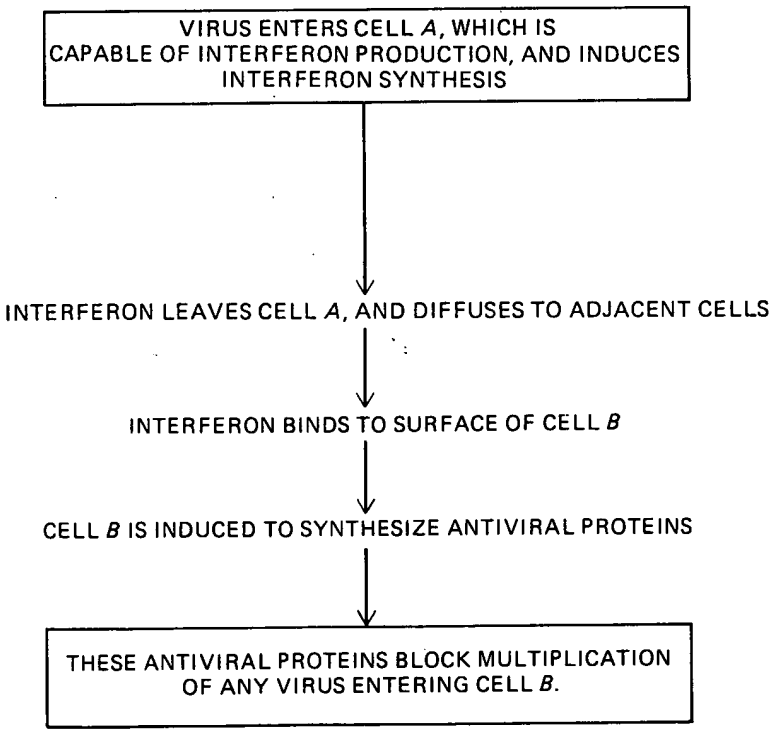


FIGURE 17-9. Role of interferon in preventing viral replication.

exciting finding in the study of interferon is that interferon exerts anticancer effects, not because cancers are caused by viruses (few, if any, human cancers are) but rather because the lymphocytes stimulated into activity by interferon attack and destroy cancer cells as well as virus-infected cells. A great deal of research is now in progress to determine whether interferon (manufactured commercially mainly by recombinant DNA techniques) can be used medically in the therapy or prevention of viral diseases and cancer.

Specific Immune Responses

The roles of lymphocytes and macrophages: An overview

Traditionally, specific immune responses are placed in one of two categories, according to the nature of the effector mechanisms employed in the response. One category has been termed **humoral** or **antibody-mediated immunity**, in recognition of the central role of circulating antibodies in the destructive process; the antibodies are secreted by **B lymphocytes** (to be more precise, by the **plasma cells** into which B lymphocytes differentiate upon being stimulated). The second category of specific immune defenses is **cell-mediated immunity**, and it is mediated not by antibodies but by intact cells, in this case largely by a second population of lymphocytes, the **T lymphocytes**, which are distinct from the B lymphocytes.

After fetal life, lymphocytes, like all leukocytes, are derived from precursor cells in bone marrow. After entering the blood, the lymphocytes take up residence in lymph nodes, thymus, spleen, and other lymphoid organs. There, upon appropriate stimulation, they undergo mitosis, so that most new lymphocytes actually arise in these lymphoid organs rather than in bone marrow. The crucial event determining whether a lymphocyte and all generations of the cells it gives rise to by mitosis will be a B cell or T cell is entry of the cell into the thymus or lack of entry.

At some point in their travels, cells destined to be T cells enter (or arise within) the thymus (thus the name T cell), which in some manner confers upon them the ability to differentiate and mature into cells competent to act as effectors in cell-mediated immunity. The T cells then leave the thymus and take residence in the other lymphoid tissues, but the thymus continues to somehow stimulate them and their offspring by means of hormones (**thymosin**) which its epithelial cells secrete. In contrast, the B cells do not enter the thymus prior to their taking up residence in lymph nodes and other peripheral lymphoid tissues.

Unlike the nonspecific defense mechanisms, B-cell and T-cell responses depend upon the cells "recognizing" the specific foreign matter to be attacked or neutralized. This recognition is made possible by the fact that molecular components of foreign cells (and other foreign matter) combine specifically with receptor sites on the surfaces of B cells and/or T cells, triggering the attack. These foreign molecular components which stimulate a specific immune response are known as **antigens**.

Most antigens are either proteins or polysaccharides. They may exist as conjugates of each other (glycoproteins) or with other substances (lipopolysaccharides or lipoproteins). An essential determinant of a molecule's capacity to serve as an antigen is size (most antigens have molecular weights of greater than 10,000). However, many low-molecular-weight substances can become attached to an already existing antigen and thereby acquire the ability to trigger a specific immune response; in such cases the small molecule is known as a **hapten**.

The B-cell system is characterized by an ability to recognize an enormous variety of different specific antigens, and its major function is to confer, via its secreted antibodies, resistance against most bacteria (and their toxins) and some viruses. By contrast, the T cells confer major resistance against cells infected by viruses or other infectious agents, some cancer cells, and solid-tissue transplants.

B cells and T cells influence each other in a variety of ways. On the one hand, antibodies (a B-cell product) may either facilitate or decrease the ability of an attacking T cell to destroy a foreign cell. On the other hand, T cells may either enhance or suppress the secretion of antibody by B cells.

This last phenomenon led to the discovery that T cells do not constitute a homogeneous population but are of three kinds: (1) **cytotoxic T cells**, which upon activation perform the role classically ascribed to T cells—the destruction of those cells listed in the previous paragraph; (2) **helper T cells**, which enhance both antibody production and cytotoxic T-cell function; and (3) **suppressor T cells**, which suppress both antibody production and cytotoxic T-cell function. As we shall see, most of the actions of T cells are mediated by chemicals which they secrete. All nonantibody chemical messengers secreted by lymphocytes (whether B or T) are collectively termed **lymphokines**; most, if not all, lymphokines are proteins.

To reiterate, it is the ability of B and T cells to recognize specific foreign antigens that confers specificity upon the immune responses in which they participate. However, another cell type we have already dealt with extensively—the macrophage—is also crucial for specific immune responses, not because it "recognizes" specific foreign antigens (it does not) but because it "cooperates" with B and T cells in a variety of essential ways (Fig. 17-10). First, macrophages must somehow "process" and "present" the foreign antigen to B cells and T cells or else the antigen will not be recognized by or activate the lymphocyte. This processing probably occurs as a result of the initial nonspecifically mediated phagocytosis carried on by macrophages that we described earlier. A second macrophage-lymphocyte interaction is that the macrophage is stimulated to secrete proteins which have profound regulatory effects on the lymphocytes. The most important of these is **interleukin 1 (IL-1)** (recall that this substance is probably identical to endogenous pyrogen), which is required for the maturation and proliferation of both B cells and T cells. Thus, the macrophages not only get the antigen to the lymphocyte, but, via IL-1, initiate a sequence of events which stimulate the now-activated lymphocyte to undergo its differentiation and multiplication.

The third macrophage-lymphocyte interaction is in the opposite causal direction; we have so far described how the macrophages influence the lymphocytes, but once fully activated and functioning, the lymphocytes induce the macrophages, as we shall see, to participate in the destruction of the foreign cells or matter which started off the entire process.

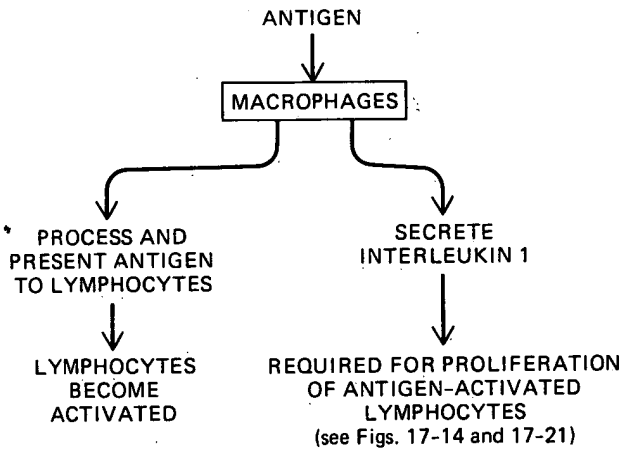


FIGURE 17-10. Effects of macrophages on lymphocytes.

Humoral immunity

Table 17-3 summarizes the sequence of events which results in antibody-directed destruction of bacteria.

Antibodies. An antibody is a specialized protein capable of combining with the specific antigen which stimulated its production. "Specific" is essential in the definition, since an antigenic substance reacts only with the type of antibody elicited by its own kind (or an extremely closely related kind) of molecule. Specificity is thus related to the chemical structure of the antigen and its antibody.

All antibodies are proteins, each composed of four interlinked polypeptide chains (Fig. 17-11). The two long chains are called heavy chains, and the two short ones are light chains (these chains are all joined by disulfide bridges). The amino acid sequences along the chains are constant from one antibody to the next except for relatively short sequences in portions of opposing light and heavy chains (Fig. 17-11). These latter sequences are

TABLE 17-3. Sequence of events in humoral immunity against bacteria

1. Bacterial antigen is carried to lymphoid tissues, where it binds to surface of specific B lymphocytes. For this to occur, macrophages must first "process" and present the antigen.
2. These B lymphocytes proliferate and differentiate into plasma cells, which secrete antibodies specific for that antigen. This is facilitated by interleukin 1, released by macrophages. It may be facilitated or suppressed by helper or suppressor T cells, respectively.
3. Antibody circulates to site of infection and combines with antigen on surface of bacteria.
4. Presence of antibody bound to antigen facilitates phagocytosis, activates complement system that further enhances phagocytosis and also directly kills bacteria, directs K cells to kill the bacteria directly.
5. Certain of the B lymphocytes differentiate into memory cells capable of responding very rapidly should the bacteria be encountered again.

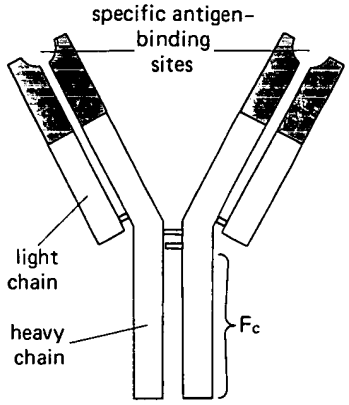


FIGURE 17-11. Antibody structure. The links represent disulfide bonds.

unique for each of the extremely large number of antibodies that an individual can produce and constitute the binding sites (two per antibody) for the antigen specific for that antibody. The constant sequences in the "stem" (or Fc portion) of the heavy chains contain nonspecific binding sites for molecules and cells which function as the effectors for antibody action (see below).

Antibodies belong to the family of proteins known as **immunoglobulins**, which may be subdivided into five classes according to the types of light and heavy chains they contain. It is very easy to misunderstand this concept, which states that there are five *classes* of immunoglobulins (antibodies), not five antibodies; each class contains many thousands of unique antibodies. These classes are designated by the letters G, A, M, D, and E after the symbol Ig (for immunoglobulin). **IgG** antibodies (also commonly called "gamma globulin"), which are the most abundant of the plasma antibodies, and **IgM** antibodies provide the bulk of specific immunity against bacteria and viruses. The other class we shall be concerned with is **IgE**, for these antibodies mediate certain allergic responses. **IgA** antibodies are secreted by the linings of the gastrointestinal, respiratory, and genitourinary tracts and exert their major activities in these secretions; they are also the major antibodies in milk. The function of the **IgD** class is presently uncertain.

Antibody production. When a foreign antigen reaches lymphoid tissues, it triggers off antibody synthesis (Table 17-3 and Fig. 17-12). The antigen may reach the spleen via the blood, but much more commonly it is carried from its site of entry via the lymphatics to lymph nodes. There it stimulates a tiny fraction of the B lymphocytes to enlarge and undergo cell division, most of the progeny of which then differentiate into **plasma cells**, which are the major antibody producers. The most striking aspect of this transformation is a marked expansion of the cytoplasm, which consists almost entirely of the granular type of endoplasmic reticulum (Fig. 17-13) found in other cells which manufacture protein for export (so all-consuming is this transformation that plasma cells die after several days of antibody production). After synthesis, the antibodies are released into the blood or lymph, which carries them

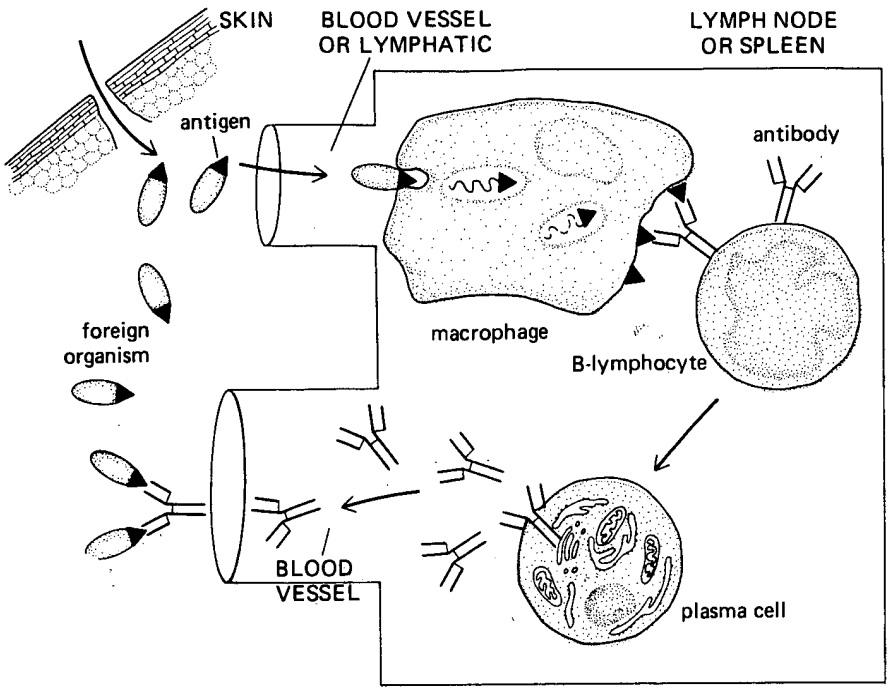


FIGURE 17-12. Induction of antibody synthesis by a microbe. The "processing" of antigen by macrophage is still not clear. The roles of helper T cells and of chemicals secreted by the macrophages are not shown (see Fig. 17-14). *(Adapted from Singer and Hilgard.)*

to the site of infection or presence of foreign matter. There the antibodies combine with the antigenic type that had elicited the antibody's production.

Some of the B-cell progeny do not fully differentiate into plasma cells but rather differentiate into "**memory**" **cells**, ready to respond rapidly should the antigen ever reappear at a future time. Thus, the presence of the memory cells will avoid much of the delay which occurs during the initial infection.

Note that we stated that only a tiny fraction of the total B cells respond to any given antigen. Different antigens stimulate entirely different populations (**clones**) of B cells. This is because the cells of any one lymphocyte clone (and the plasma cells it gives rise to) are capable of secreting only one kind of antibody. Thus, according to this clonal theory, different antigens do not direct a single cell to produce different antibodies; rather each specific antigen triggers activity in the clone of cells already predetermined to secrete only antibody specific to that antigen. The antigen selects this particular clone and no other because each B cell has immunoglobulin binding sites on its plasma membrane similar to those of the antibodies which it is capable of producing (after differentiation into a plasma cell). These surface immunoglobulins act as receptor sites with which the antigen can combine, triggering off the entire process of division, differentiation, and antibody secretion just described. The staggering but statistically possible implication is that there must exist millions of different clones, at least one for each of the possible antigens an individual *might* encounter during life.

It must be emphasized that interaction between antigen and B-cell receptor site is considerably more complex than that just described, for macrophages also play a crucial role (Fig. 17-14). During antigen activation of the B cells, there occurs a clustering of macrophages around the relevant B-

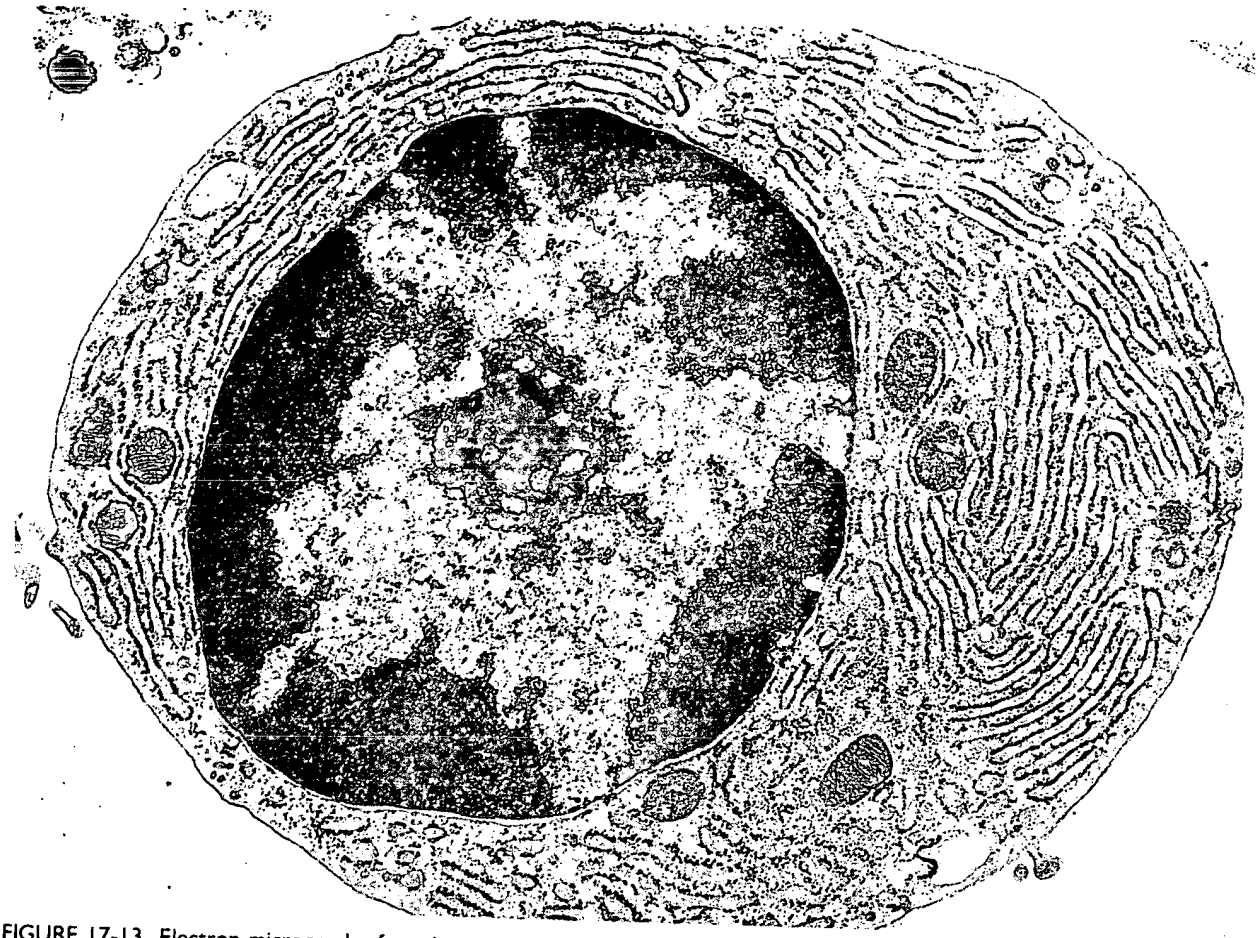


FIGURE 17-13. Electron micrograph of a guinea pig plasma cell. Note the extensive endoplasmic reticulum. *(From W. Bloom and D. W. Fawcett, "A Textbook of Histology," 9th ed., W. B. Saunders Company, Philadelphia, 1968.)*

cell clone. The macrophages "process" and "present" the antigen in some way so as to allow it to activate the B-cell receptor sites. In addition to presenting the antigen to the B cell, the macrophages secrete interleukin 1 (IL-1), which stimulates proliferation of the antigen-activated B cells.

For many antigens, only the interaction between macrophage and B lymphocyte is required to initate B-cell proliferation, differentiation, and antibody secretion. However, for others, participation of **helper T cells** is also essential. In these cases, the antigen processed by the macrophage is presented in such a way that helper T cells, as well as B cells, are activated by it. One way the activated helper T cell stimulates the B cell is via its secretion of a chemical (**B-cell growth factor**) which acts in concert with interleukin 1. In some cases, **suppressor T cells**, which inhibit B-cell function, are brought into play; these suppressor T cells provide an important dampening effect when antibody production is becoming excessive.

Functions of antibodies. In a previous section, we described how a local inflammatory response is induced nonspecifically by any tissue damage. Now we shall see that the presence of antigen-antibody complexes triggers events which profoundly *amplify* the inflammatory response. In other words,

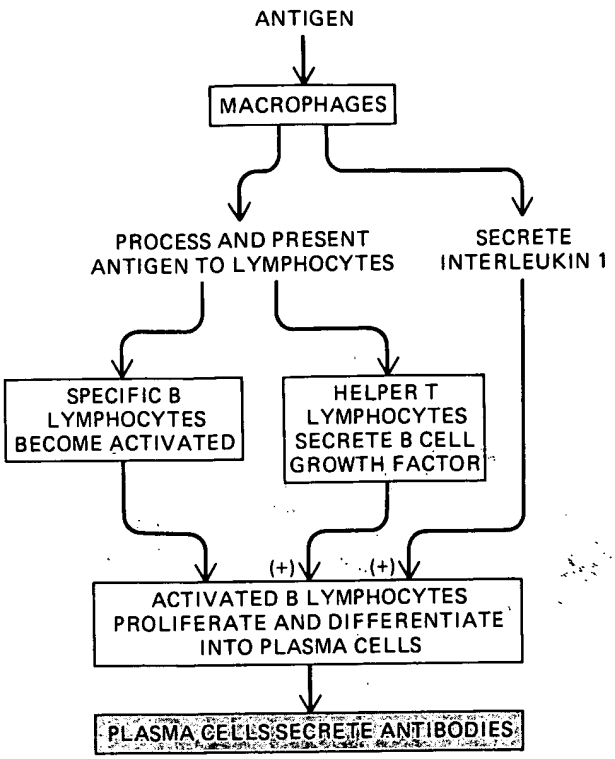


FIGURE 17-14. Activation, proliferation, and differentiation of B lymphocytes.

the major function of humoral immune mechanisms is to enhance and make more efficient the inflammatory response and cell elimination already initiated in a nonspecific way by the invaders. Thus, vasodilation, increased vascular permeability to protein, neutrophil exudation, phagocytosis, and killing of microbes without prior phagocytosis are all markedly enhanced.

Activation of complement system. There are several mechanisms by which the presence of antigen-antibody complexes enhances inflammation, but the single most important involves the complement system. As described earlier, this system, which generates not only terminal proteins of the membrane attack complex that directly kills microbes, but mediators of virtually every process in inflammation, is involved in the nonspecific inflammatory response. However, the presence of antibody attached to antigen increases its participation because the antibody-antigen complex is a powerful activator of the first step in the so-called **classical complement pathway** (Fig. 17-15). The first molecule in this pathway, C_1, binds to complement receptors on the Fc (stem) portions of the antibody to which antigen has become complexed (Fig. 17-16), and this binding activates the enzymatic portions of C_1, thereby initiating the entire complement pathway leading through cleavage of C_3 and on to the formation of the membrane attack complex (Fig. 17-5).

It is important to note that C_1 binds not to the unique antigen-specific binding sites in the antibody's "prongs" but rather to binding sites in the antibody's stem (Fig. 17-11). Since the latter are the same in virtually all antibodies of the IgG and

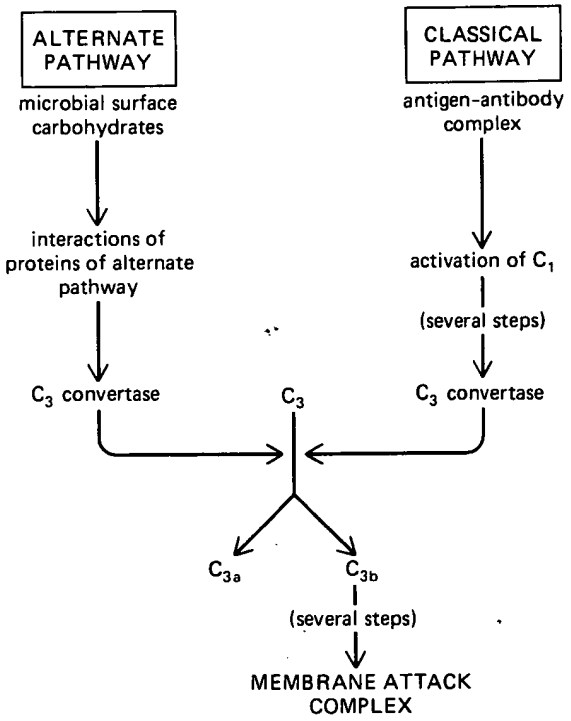


FIGURE 17-15. Alternate and classical pathways for generating C_3 convertase (the C_3 convertases in the two pathways have different structures, but both cleave C_3). Once C_3 is cleaved, the pathways are identical.

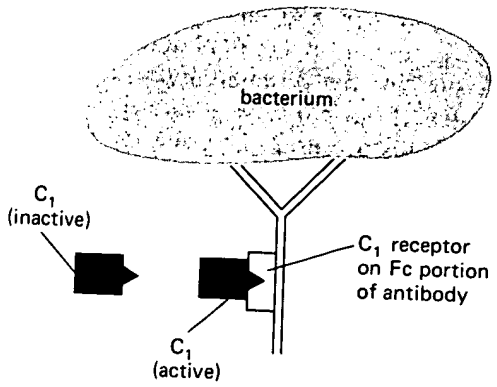


FIGURE 17-16. Initiation of classical complement pathway. The enzymatic ability of C_1 is activated by its binding to the Fc (stem) portion of an antibody, itself bound to specific antigen. Active C_1 then activates the next complement molecule in the pathway while attached to the antibody. The structures in the figure are not drawn to scale.

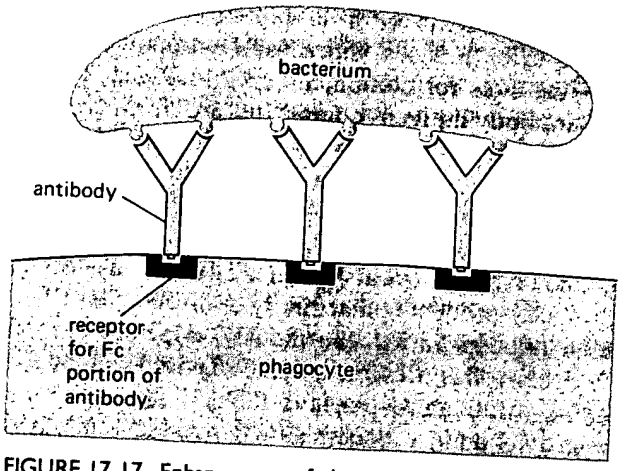


FIGURE 17-17. Enhancement of phagocytosis by antibody. The antibody links the phagocyte to the bacterium.

IgM classes, the complement molecule will bind to *any* antibodies belonging to these classes. In other words, there is only one set of complement molecules, and once activated, they do essentially the same thing, regardless of the specific identity of the invader. In contrast, the formation of antibodies to antigens on the invader and their subsequent combination are highly specific.

To reiterate, the function of the antibodies is to "identify" the invading cells as foreign by combining with antibody-specific antigens on the cell's surface. The complement system is subsequently activated when the first complement molecules in the sequence combine with this antigen-bound antibody, and the cascade of activated complement molecules then mediates the actual attack.

Direct enhancement of phagocytosis. Activation of complement is not the only mechanism by which antibodies enhance phagocytosis. Merely the presence of antibody attached to antigen on the microbe's surface has some enhancing effect, i.e., antibody acts as an opsonin.

The mechanism is analogous to that for complement in that the antibody links the phagocyte to the antigen. As shown in Fig. 17-17, the phagocyte has receptors on its surface to which bind the Fc (stem) portion of antibodies. This linkage promotes attachment of the antigen to the phagocyte and the triggering of phagocytosis.

Activation of K cells. The presence of antibody attached to specific antigens on a foreign cell brings into play not only phagocytes but a little-understood population of lymphocytes known as **K cells** (for **killer cells**). These cells also have receptors for the Fc portion of antibodies and so are brought into intimate contact with foreign cells via this linkage (in Fig. 17-17, all one would have to do is change "phagocyte" to "K cell"). K cells, however, do not phagocytize the foreign cells but rather kill them directly by lysing their outer membranes.

Direct neutralization of bacterial toxins and viruses. Bacterial toxins and certain viral components act as antigens to induce antibody production. The antibodies then combine with the toxins and viruses to "neutralize" them. Neutralization in reference to a virus means that the combined antibody prevents attachment of the virus to host cell membranes, thereby preventing virus entry into the cell. Similarly, antibodies neutralize bacterial toxins by combining with them, thus preventing the interac-

tion of the toxin with susceptible cell-membrane sites. In both cases, since each antibody has two binding sites for combination with antigen, chains of antibody-antigen complexes are formed (Fig. 17-18) which are then phagocytized.

Active and passive humoral immunity. We have been discussing antibody formation without regard to the course of events in time. The response of the antibody-producing machinery to invasion by a foreign antigen varies enormously, depending upon whether it has previously been exposed to that antigen. Antibody response to the first contact with a microbial antigen occurs slowly over several days, with some circulating antibody remaining for long periods of time, but a subsequent infection elicits an immediate and marked outpouring of additional antibody (Fig. 17-19). This represents a type of "memory" which converts a greatly enhanced resistance toward subsequent infection with that particular microorganism. This resistance, built up as a result of actual contact with microorganisms and their toxins or other antigenic components, is known as **active immunity**. Until modern times, the only way to develop active immunity was actually to suffer an infection, but now a variety of other medical techniques are used, i.e., the injection of **vaccines**, or microbial derivatives. The actual material injected may be small quantities of living or dead microbes, e.g., polio vaccine, small quantities of toxins, or harmless antigenic materials derived from the microorganism or its toxin. The general principle is always the same: Exposure of the body to the agent results in the induction of the antibody-synthesizing machinery required for rapid, effective response to possible future infection by that particular organism. However, for many microorganisms (the common cold virus, for example), the memory component of the antibody response does not occur, and antibody formation follows the same time course regardless of how often the body has been infected with the particular microorganism.

A second kind of immunity, known as **passive immunity**, is simply the direct transfer of actively

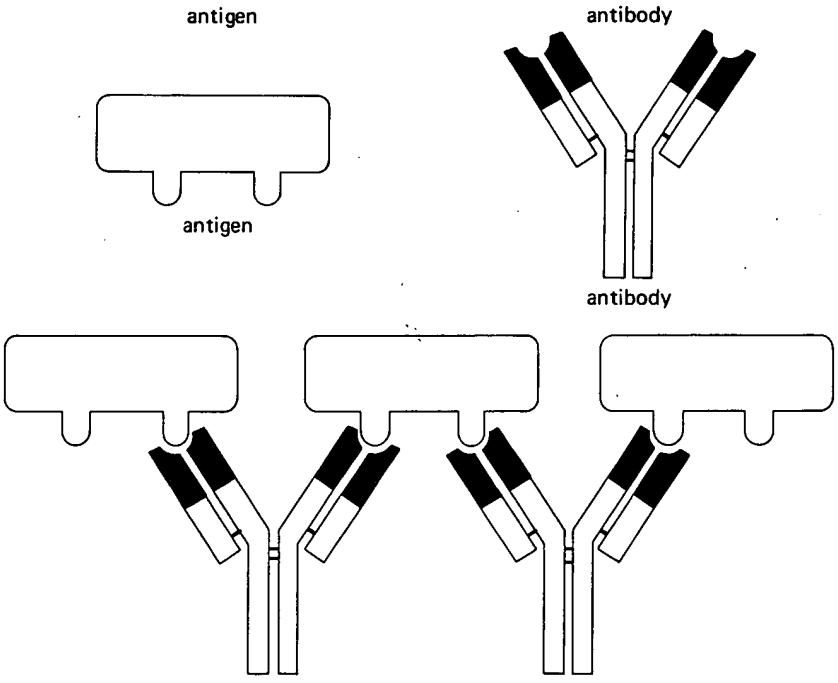


FIGURE 17-18. Interlocking complex of antigens and antibodies.

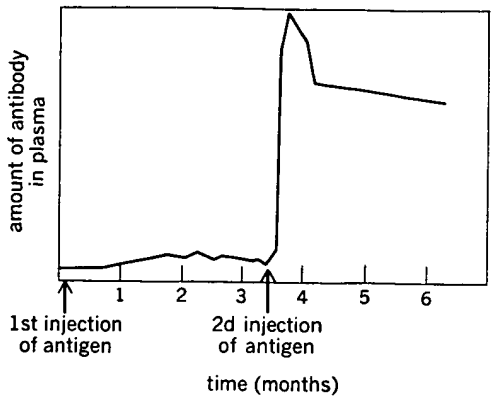


FIGURE 17-19. Rate of antibody production following the initial contact with an antigen and a subsequent contact with the same antigen.

formed antibodies from one person (or animal) to another, the recipient thereby receiving preformed antibodies. Such an exchange (for IgG) normally occurs between fetus and mother across the placenta. Breast-fed children also receive antibodies (of the IgA class) in the mother's milk. These are important sources of protection for the infant during the first months of life, when the antibody-synthesizing capacity is relatively poor.

The same principle is used clinically when specific antibodies or pooled gamma globulin is given a person exposed to or actually suffering from certain infections, such as measles, hepatitis, or tetanus. The protection afforded by this transfer of antibodies is relatively short-lived, usually lasting only a few weeks. The procedure is not without danger since the injected antibodies (often of nonhuman origin) may themselves serve as antigens, eliciting antibody production by the recipient and possibly severe allergic responses.

Summary. We may now summarize the interplay between nonspecific and specific humoral immune mechanisms in resisting a bacterial infection. When a bacterium is encountered for the first time, *nonspecific* defense mechanisms resist its entry and, if entry is gained, attempt to eliminate it both by phagocytosis and, to some extent, by nonphagocytic killing. Simultaneously, the bacterial antigens induce the differentiation of specific B-cell clones into plasma cells capable of antibody production. If the nonspecific defenses are rapidly successful, these slowly developing *specific* immune responses may never play an important role. If the nonspecific defenses are only partly successful, the infection may persist long enough for significant amounts of antibody to reach the scene; the presence of antibody, in a variety of ways, leads to both enhanced phagocytosis and direct destruction of the foreign cells. In either case, all subsequent encounters with that type of bacteria will be associated with the same sequence of events, with the crucial difference that the specific humoral immune responses may be brought into play much sooner and with greater force; i.e., the person might have active immunity against that type of bacteria.

Cell-mediated immunity

Cytotoxic T cells. The cytotoxic T lymphocytes are responsible for highly specific cell-mediated immunity. Individual T cells, like B cells, are clonal in that each T cell bears on its plasma membrane genetically determined receptor sites for a specific foreign antigen. These specific surface receptors are similar to immunoglobulins. In contrast to B cells, cytotoxic T cells will (with one major exception) ignore a foreign antigen unless it is present on a cell surface which also contains an antigen identical to one of the T cell's own "self-antigens."

Each of us has a group of genes (the **major histocompatibility complex** or **MHC**) which codes for many proteins important for immune function. Among these are a group of proteins located on the plasma membranes of all nucleated cells in an individual's body and called **human leukocyte associated antigens (HLA antigens)** (the name reflects the fact that these proteins were first discovered in leukocytes; they are also called **histocompatibility antigens**). Since no two persons (other than identical twins) have the same MHC genes, no two persons have the same HLA antigens. To restate the earlier generalization: T cells will be activated to attack a cell which bears on its surface a foreign antigen *plus* an antigen identical to one of the T cell's own HLA antigens. As we shall see, this is the situation

which exists for several categories of the cytotoxic T cell's favorite targets—viral-infected host cells and cancer cells—and explains why the cytotoxic T cells have a much more restricted set of targets than do the B cells.

Upon initial exposure to the appropriate antigen, which must first be "processed" by macrophages, the cytotoxic T cell becomes "sensitized"; i.e., it undergoes, over 1 to 2 weeks, enlargement and mitosis, with differentiation of the daughter cells. So far, the story sounds similar to that described earlier for B cells, but the crucial difference has to do with the activities of these differentiated daughter cells: Instead of secreting antibodies (as do the plasma-cell progeny of B cells), these sensitized cytotoxic T cells actually combine with the foreign antigen on the surface of the cell being attacked (Fig. 17-20), and this triggers the release by the cytotoxic T cell of one or more chemicals which lyse the attached cell's plasma membrane, thus kill-

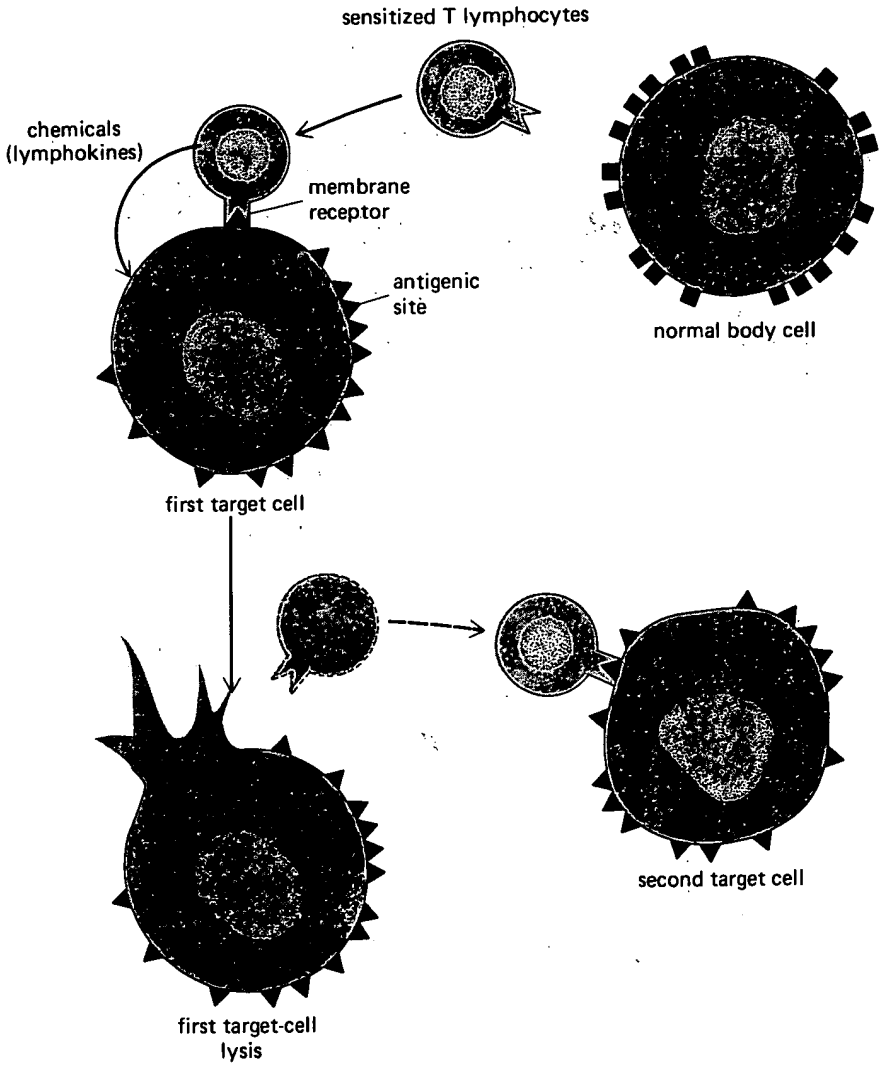


FIGURE 17-20. Killing of foreign (target) cells by sensitized cytotoxic T lymphocytes. The killing of the target cell is achieved by chemicals (lymphokines) secreted by the sensitized T cell. *(Adapted from Cerottini.)*

ing it directly (i.e., without prior phagocytosis). We must emphasize the important geographic difference between plasma cell and sensitized T-cell function: Antibodies are secreted by plasma cells located in lymph nodes and other lymphoid organs often far removed from the invasion site and reach the site via the blood; in contrast, a sensitized cytotoxic T cell travels to the invasion site where it combines with foreign antigen on the cell under attack and kills it.

As is true for the B system, some of the sensitized T cells do not actually participate in the immune response but serve as a "memory bank" which greatly speeds up and enhances the immune response if the person is ever exposed to the specific antigen again. Thus, active immunity exists for cell-mediated immune responses just as for antibody responses. Passive immunity also exists in this system and can be conferred by administering sensitized lymphocytes taken from a previously infected person (or animal).

Cytotoxic T cells are not the only class of T cells which participate in cell-mediated immunity. Helper and suppressor T cells importantly regulate the cytotoxic T cells, just as they do B cells in humoral immunity. For one thing, helper T cells secrete a lymphokine known as **interleukin 2 (IL-2)**, which stimulates the proliferation of antigen-activated T cells (thus, IL-2 does for T cells what B-cell growth factor—also secreted by helper T cells—does for B cells). This secretion of IL-2 is, itself, stimulated by interleukin 1 released from macrophages (Fig. 17-21).

But IL-2 is not the only chemical messenger secreted by helper T cells in cell-mediated immune responses. They also secrete proteins which act as an amplification system for the facilitation of the inflammatory response and killing by macrophages (Fig. 17-21). Thus, cell-mediated immunity is analogous to humoral immunity in that it serves, in large part, to enhance and make more efficient the nonspecific defense mechanisms already elicited by the foreign material. The major difference is that humoral immunity utilizes a circulating group of plasma proteins (the complement system) as its major amplification system, whereas the T cells literally produce and secrete their own chemical amplification system.

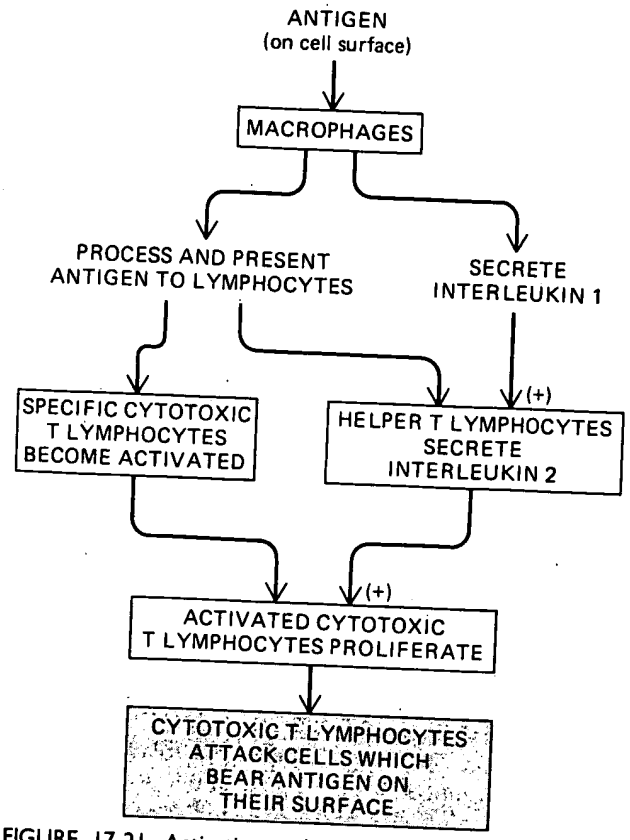


FIGURE 17-21. Activation and proliferation of cytotoxic T cells. Compare to Fig. 17-14. Not shown in the figure is the fact that interleukin 2 also causes helper T cells to proliferate, and secrete many lymphokines which facilitate inflammation.

As might be predicted, some of these chemicals are chemotaxins. These serve to attract some neutrophils but many more monocytes to the area. The monocytes are converted to macrophages and begin their job of phagocytosis. The T cells not only secrete chemotaxins to attract the macrophages-to-be but they also secrete another substance which keeps the macrophages in the area and stimulates them to greater activity (indeed, such revved-up macrophages are known as "angry" macrophages). Finally, interferon is also released in large quantity from activated T cells; as mentioned earlier, this substance not only exerts inhibitory effects on viral

replication but markedly enhances the killing action of cytotoxic T cells.

Natural killer (NK) cells. Our description of specific cell-mediated immunity has been restricted thus far to T cells. Recently, another population of cells, known as **natural killer (NK) cells** has been discovered which have certain properties similar to those of cytotoxic T cells, namely, they combine with foreign antigens on their target cells and kill them directly (by lysing their membranes). Thus, to visualize NK cell action, just substitute "NK cell" for "sensitized T cell" in Fig. 17-20. Moreover, as is true for cytotoxic T cells, major targets for NK cells are tumor cells and virus-infected host cells.

But NK cells (which may be yet another subset of lymphocytes) are definitely not cytotoxic T cells, from which they differ in important ways. First, they are not specific for only one kind of foreign antigen; rather there are a relatively few clones of NK cells, each of which can recognize and bind to a spectrum of different foreign antigens (neither the binding sites on NK cells nor the nature of the antigens on the target cells are known). Second, and very important, NK cells do not need prior exposure to antigen followed by a long maturation period before they can function (this is why they are called "natural" killers). Thus NK cells provide (along with the completely nonspecific mechanisms described earlier in this chapter) an immediately responding first line of defense against cancer cells, virus-infected host cells, and perhaps other microbes as well.

A central role in the function of NK cells is played by interferon. Activated NK cells (like activated T cells) secrete large quantities of interferon. This interferon then not only inhibits viral replication but also markedly enhances the killing actions of the NK cells (just as it does those of cytotoxic T cells and macrophages). Thus interferon and NK cells operate in a positive-feedback manner (Fig. 17-22).

One more point about NK cells concerns their relationship to K cells, the lymphocyte subset which kills cells directly when guided to do so by antibodies. NK cells do not require antibodies to kill their target cells, and so it has been presumed that K and NK cells are distinct cells. It now seems much more likely that they are, in fact, one and the same cell, and whether antibody influences their activity depends upon the milieu in which the attack occurs.

Cell-mediated defenses against viral infection. The body's defenses against viral infection (summarized in Table 17-4) include virtually all the processes we have been describing. It should be clear from Table 17-4 that the host defense mechanisms against viruses operate in two spheres: (1) when the virus is still in the extracellular fluid; and (2) after the virus has taken up residence within the body's cells (something it must do to survive and replicate; replicated virus may leave the original host cell and once again enter the extracellular fluid to seek out new host cells).

The specific immune defense against intracell-

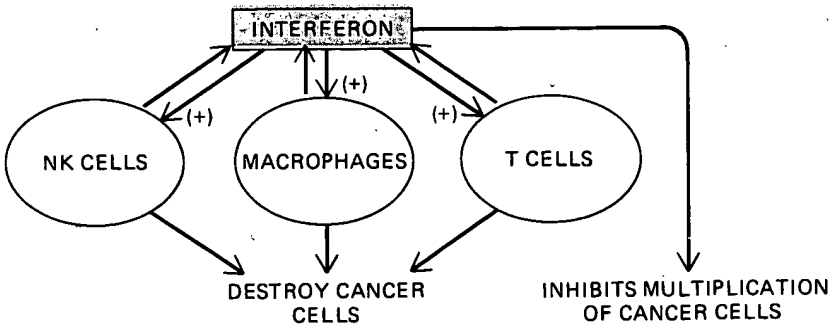


FIGURE 17-22. Anticancer effects of interferon. All three of the cell types which directly attack cancer cells secrete interferon, which then enhances the ability of the cells to destroy cancer cells and also, itself, inhibits multiplication of cancer cells.

TABLE 17-4. Summary of host responses to viruses

	Main cells involved	Comment on action
Nonspecific responses		
Anatomic barriers	Surface linings of body	Simple barrier; antiviral chemicals
Inflammation	Tissue macrophages	Phagocytosis of extracellular virus
Interferon	Multiple cell types	Prevention of viral replication inside host cells
Specific responses		
Humoral immunity	Plasma cells derived from B lymphocytes secrete antibodies that perform the actions listed to the right	Neutralize virus and thus prevent entry to cell Activate complement that leads to both enhanced phagocytosis and direct destruction of extracellular virus
Cell-mediated immunity	Sensitized T lymphocytes and NK cells secrete chemicals that perform the actions listed to the right	Destroy host cell and thus induce release of virus so that it can be phagocytized Prevent viral replication (the chemical mediating this effect is interferon.)

ular virus is cell-mediated, by cytotoxic T lymphocytes and by NK cells. In order to attack a virus once it has gained cellular entry, the T cell or NK cell must destroy the cell itself. Such destruction occurs because the viral nucleic acid codes for a plasma-membrane protein foreign to the body, and once this protein has been synthesized by the host cell, it can act as an antigen to elicit a T-cell or NK cell attack. (Recall that cytotoxic T cells "recognize" and attack a cell bearing foreign antigen on its surface when the surface also contains "self-antigens" of the HLA group—clearly the case in viral-infected host cells.) The result is destruction of the host cell with release of the viruses, which can then be directly attacked. Generally, only a few host cells must be sacrificed in this way, but once viruses have had a chance to replicate and spread from cell to cell, so many host cells may be attacked by the body's own defenses that serious malfunction may result.

Immune surveillance: Defense against cancer. Another function of cell-mediated immunity is to recognize and destroy cancer cells. This is made possible by the fact that virtually all cancer cells have some surface antigens different from those of other body cells and can, therefore, be recognized as "foreign." It is likely that cancer arises as a result of genetic alteration (by chemicals, radiation, etc.) in previously normal body cells. One manifestation of the genetic change is the appearance of the new surface antigens (side by side with the cell's own HLA antigens). Circulating T cells encounter and become sensitized to these cells, combine with the antigens on their surface, and release the chemicals which destroy the cells directly; they also call in attacking macrophages.

As mentioned earlier, NK cells also participate in the attack on cancer cells. Indeed, because they do not require a long period of sensitization, they are the single most important effector cell in immune surveillance. Note also (Fig. 17-22) that all three of the cell types which directly attack cancer cells secrete interferon, which, in turn, stimulates the cytotoxicity of these cells.

There occur important interactions between the humoral and cell-mediated systems, one of which actually protects the cancer cell. We have pointed out that the cancer-specific antigens stimulate the development of sensitized T cells against the cancer cells. Simultaneously, they may also stimulate the production by B cells of circulating antibodies (of the IgG class). These antibodies combine with the cell-surface antigen sites, and the next events may be quite variable; in some cases, the antibody may facilitate the destruction of the cancer cell, whereas in most cases it may actually protect the cell by preventing T cells from combining with the antigen. In the latter cases, the antibodies are called "blocking" antibodies and the process is

called **immune enhancement**. Whether facilitation or blocking occurs seems to depend upon precisely how the antibody interacts with the antigen, but many unanswered questions remain (for example, why do the blocking antibodies not activate complement which would destroy the cancer cells?).

Rejection of tissue transplants. The cell-mediated immune system is also mainly responsible for the recognition and destruction, i.e., **rejection**, of tissue transplants (Fig. 17-23). As described earlier, on the surfaces of all nucleated cells of an individual's body are genetically determined antigenic protein molecules known as HLA or histocompatibility antigens. When tissue is transplanted from one individual to another, those surface antigens which differ from the recipient's are recognized as foreign and are destroyed by sensitized cytotoxic T cells. (This constitutes the one exception to the generalization that cytotoxic T cells will attack only cells which bear on their surface one of the T cell's own HLA antigens side by side with foreign antigen.) As is true for the response to cancer cells, the foreign cells may also stimulate the secretion of circulating "blocking" antibodies; if this occurs, the chances for graft survival are enhanced. Note that, in immunology, whether a phenomenon is desirable or undesirable depends on the point of view; cancer enhancement is undesirable whereas graft enhancement is desirable.

Some of the most valuable tools aimed at reducing graft rejection are radiation and drugs which kill actively dividing lymphocytes and, thereby, decrease the T-cell population. Unfortunately, this also results in depletion of B cells as well, so that antibody production is diminished and the patient becomes highly susceptible to infection. A more discriminating method presently being tried is to prepare and inject into the recipient antibodies against the T cells; by this means, the T cells would

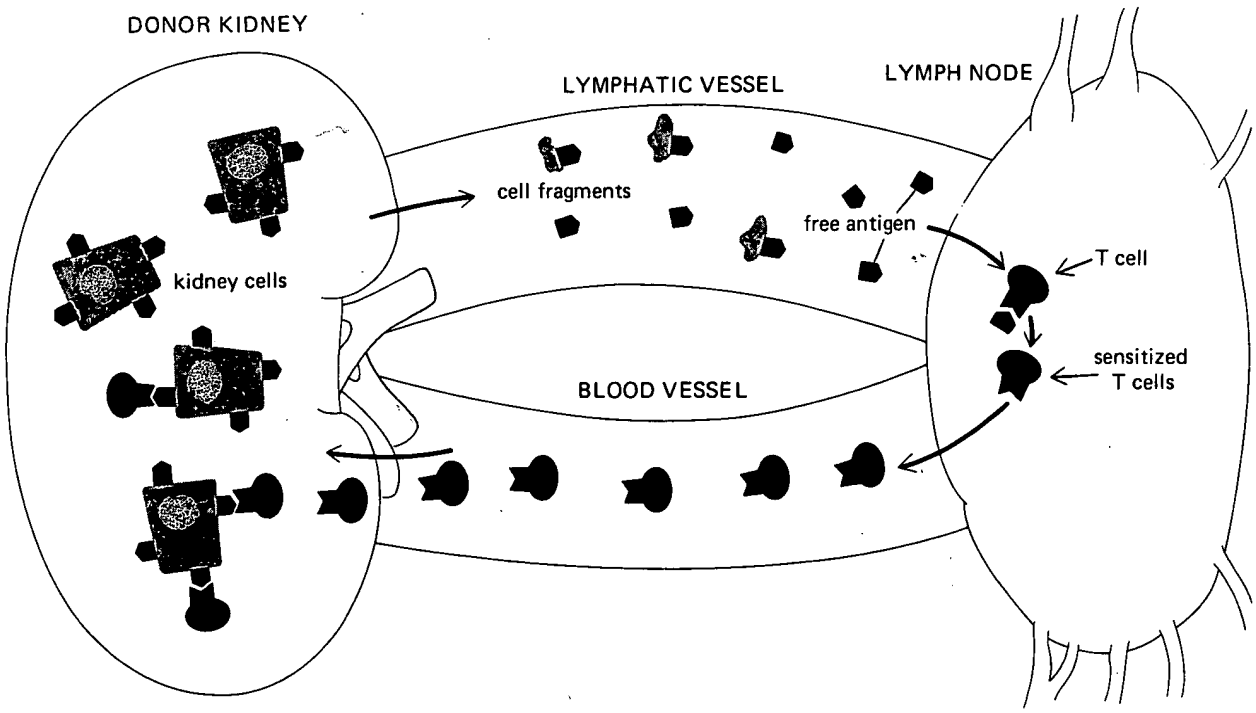


FIGURE 17-23. Mechanism of rejection of renal transplant by sensitized cytotoxic T cells. The roles of macrophages in antigen processing, T-cell sensitization, and kidney-cell destruction are not shown. Nor is the role of helper T cells. *(Adapted from Hume.)*

be destroyed but not the B cells. Recently, an extremely effective drug (cyclosporin) has come into use which blocks either the production or action of interleukin 2, the substance produced by helper T cells which is required for proliferation of antigen-activated cytotoxic T cells; this drug leaves the B-cell system essentially untouched.

Transfusion Reactions and Blood Types

Transfusion reactions are a special example of tissue rejection, one which illustrates the fact that antibodies rather than sensitized T cells can sometimes be the major factor in leading to the destruction of nonmicrobial cells. Among the large number of erythrocyte membrane antigens, we still recognize those designated A, B, and O as most important. These antigens are inherited, A and B being dominant. Thus, an individual with the genes for either A and O or B and O will develop only the A or B antigen. Accordingly, the possible blood types are A, B, O, and AB. If the typical pattern of antibody induction were followed, one would expect that a type A person would develop antibodies against type B cells only if the B cells were introduced into the body. However, what is atypical of this system is that even without initial exposure, the type A person always has a high plasma concentration of anti-B antibody. The sequence of events during early life which lead to the presence of the so-called **natural antibodies** in all type A persons is unknown. Similarly, type B persons have high levels of anti-A antibodies; type AB persons obviously have neither anti-A nor anti-B antibody; type O persons have both; anti-O antibodies are usually not present in anyone.

With this information as background, what will happen if a type A person is given type B blood? There are two incompatibilities: (1) The recipient's anti-B antibody causes the transfused cells to be attacked; and (2) the anti-A antibody in the transfused plasma causes the recipient's cells to be attacked. The latter is generally of little consequence, however, because the transfused antibodies become so diluted in the recipient's plasma that they are ineffective. It is the destruction of the transfused cells which produces the problems. The range of possibilities is shown in Table 17-5. It should be evident why type O people are frequently called "universal donors" whereas type AB people are "universal recipients." These terms, however, are misleading and dangerous since there are a host of other erythrocyte antigens and plasma antibodies besides those of the ABO type. Therefore, except in dire emergency, the blood of donor and recipient must be carefully matched.

Another antigen of medical importance is the so-called **Rh factor** (because it was first studied in rhesus monkeys) now known to be a group of erythrocyte membrane antigens. The Rh system follows the classic immunity pattern in that no one develops anti-Rh antibodies unless exposed to Rh-type cells (usually termed Rh-positive cells) from another person. Although this can be a problem in an Rh-negative person, i.e., one whose cells have no Rh antigen, subjected to multiple transfusions with Rh-positive blood, its major importance is in the mother-fetus relationship. When an Rh-negative mother carries an Rh-positive fetus, some of the fetal erythrocytes may cross the placental barriers into the maternal circulation, inducing her to synthesize anti-Rh antibodies. Because the movement of fetal erythrocytes into the maternal circulation occurs mainly during separation of the placenta at delivery, a first Rh-positive pregnancy rarely offers any danger to the fetus, since delivery occurs before the antibodies can be made. In future pregnan-

TABLE 17-5. Summary of ABO blood-type interactions

Recipient	Donor	Compatible ?
A	O, A	yes
B	O, B	yes
AB	O, A, B, AB	yes
O	O	yes
A	AB, B	no
B	AB, A	no
AB	— — —	— — —
O	AB, A, B	no

cies, however, these anti-Rh antibodies will already be present in the mother and can cross the placenta to attack the erythrocytes of an Rh-positive fetus. The risk increases with each Rh-positive pregnancy as the mother becomes more and more sensitized. Fortunately, Rh disease can be prevented by giving any Rh-negative mother gamma globulin against Rh erythrocytes within 72 h after she has delivered an Rh-positive infant. These exogenous antibodies bind to the antigenic sites on any Rh erythrocytes which might have entered the mother's blood during delivery and prevent them from inducing antibody synthesis by the mother (both the cells and exogenous antibody are soon destroyed).

Factors Which Alter the Body's Resistance to Infection

Let us examine two seemingly opposed statements: (1) Tuberculosis is caused by the tubercle bacillus; (2) tuberculosis is caused by malnutrition. The first statement seems the more accurate in the sense that the disease, tuberculosis, will not occur in the absence of infection by tubercle bacilli. Yet we also [illegible] that many people harbor these bacteria, but [illegible] elop tuberculosis. There must exist, the[illegible] other factors which, by upsetting the balance between host and microbe, permit invasion, multiplication, and production of symptoms. In a sense, then, malnutrition "causes" tuberculosis by doing just this. In fact, there need be no quibbling over semantics once one realizes that the presence of the microbe is the necessary but frequently not sufficient cause of the disease. Therefore, it becomes very important to define those influences which determine the body's capacity to resist infection (as well as cancer cells and transplants). We offer here only a few examples.

Nutritional status is extremely important, and **protein-calorie malnutrition** is, worldwide, the single greatest contributor to decreased resistance to infection. The role that excesses or deficiencies of individual nutrients play in altering resistance to infection is now receiving much study, and no simple conclusions can be made. For example, severe iron deficiency predisposes to infection, but so does an excess of iron.

A preexisting disease (infectious or noninfectious) can also predispose the body to infection. Diabetics, for example, suffer from a propensity to numerous infections, at least partially explainable on the basis of defective leukocyte function. Moreover, any injury to a tissue lowers its resistance, perhaps by altering the chemical environment or interfering with blood supply.

It is also likely (although this point remains controversial) that a person's state of mind (whether the person is stressed, happy, depressed, etc.) can influence his resistance to infection; the physiological links between "state of mind" and resistance remain to be determined (one possibility will be presented in the section of this chapter dealing with "stress").

In numerous examples one of the basic resistance mechanisms itself is deficient. A striking case is that of congenital deficiency of plasma gamma globulin, i.e., failure to synthesize antibodies; these patients cannot survive without either frequent intravenous injections of gamma globulin or isolation from microbes. Similarly, a variety of complement deficiencies exist. A decrease in the production of leukocytes is also an important cause of lowered resistance, as, for example, in patients given drugs specifically to inhibit rejection of tissue or organ transplants. The total quantity of leukocytes circulating is not necessarily critical. Patients with leukemia, for example, may have tremendous numbers of blood neutrophils or monocytes, but these cells are almost all immature or otherwise incapable of normal function; such patients are extremely prone to infection. Reduced functional activity of lymphocytes is also seen in the elderly and may account for their decreased resistance.

One of the most striking examples of the lack of a basic resistance mechanism is the disease called **acquired immune deficiency syndrome (AIDS)**. This disease is characterized by profound inability to resist many infections. The major defect is a lack of helper T cells, caused by destruction or alteration of these cells by certain viruses. Clinical trials are underway to determine whether the

administration to these patients of interleukin 2, a major lymphokine secreted by helper T cells, can result in improvement.

Finally, we must mention the most important of the external agents we employ in altering resistance to infection—the **antibiotics**, such as penicillin. Antibiotics exert a wide variety of effects, but the common denominator is interference with the synthesis of one or more of the bacteria's essential macromolecules. Use of these antibacterial agents is made possible because they are harmful to microbes but relatively innocuous to the body's cells. This characteristic distinguishes them from the **disinfectants**, which are highly effective antibacterial agents but equally toxic to the body. The word "relatively" is quite important, since all antibiotics are toxic to a lesser or greater degree and must not be used indiscriminately. A second reason for judicious use is the problem of drug resistance. Most large bacterial populations contain mutants which are not sensitive to the drug and which are thus selected out by the drug. These few are capable of multiplying into large populations resistant to the effects of that particular antibiotic. Perhaps even more important, resistance can be transferred from one microbe directly to another previously nonresistant microbe by means of chemical agents ("**resistance factors**") passed between them. A third reason for the judicious use of antibiotics is that these agents may actually contribute to a new infection by altering the normal flora so that overgrowth of an antibiotic-resistant species occurs.

Allergy (Hypersensitivity Reactions)

A certain portion of the population is capable of acquiring specific-immune reactivity to environmental antigens such as dusts, pollens, food constituents, etc. Despite the relative harmlessness of most of these antigens, subsequent exposure to them elicits an immune attack which produces distressing symptoms and, often, outright bodily damage. This phenomenon, known as **allergy** or **hypersensitivity**, is in essence immunity gone wrong, for the response is really inappropriate to the stimulus.

Depending upon the antigen, allergic responses may be due to activation of either the humoral (antibody) or cell-mediated systems. In the latter cases (the skin rash after contact with poison ivy is an example), the inflammatory response is simply a T-cell–mediated affair of the kind we have discussed; because it takes several days to develop, it is known as **delayed hypersensitivity**. In contrast, antibody-mediated hypersensitivity responses are usually *immediate* in onset and involve a group of antibodies and a sequence of events different from the classical antibody-mediated response to bacteria.

Immediate hypersensitivity

Initial exposure to the antigen in the immediate-hypersensitivity type of allergy leads to some antibody synthesis but, more important, to the memory storage which characterizes active immunity. Upon reexposure, the antigen elicits a more powerful antibody response. So far, none of this is unusual, but the fact is that these particular antigens stimulate the production of the **IgE** class of antibodies. Upon their release from plasma cells, these antibodies circulate to various parts of the body and attach themselves to **mast cells**. When the antigen then combines with the IgE attached to the mast cell, this triggers release of the mast cell's secretory vesicles which contain **histamine**, other vasoactive chemicals, and chemotaxins specific for **eosinophils**. In addition to releasing these preformed inflammatory mediators stored in mast-cell vesicles, the binding of IgE to receptors triggers the mast cell to synthesize **prostaglandins** and related substances (Chap. 7), which exert many inflammation-stimulating effects. All these chemicals then initiate a local inflammatory response. Thus, the symptoms of antibody-mediated allergy are due to the various effects of these chemicals and the body site in which the antigen–IgE–mast-cell combination occurs. For example, when a previously sensitized person inhales ragweed pollen, the antigen combines with IgE-mast cells in the respiratory passages. The chemicals released cause increased mucus secretion, increased blood flow, leakage of protein, and contraction of the smooth muscle lining the airways. (**Leukotrienes** are particularly po-

tent in inducing this last effect.) Thus, there follow the symptoms of congestion, running nose, sneezing, and difficulty in breathing which characterize hayfever.

In this manner, the allergic symptoms may be localized to the site of entry of the antigen. However, if very large amounts of the chemicals released enter the circulation, systemic symptoms may result and cause severe hypotension and bronchiolar constriction. This sequence of events (**anaphylactic shock**) can actually cause death (due to circulatory and respiratory failure) and can be elicited in some sensitized people by the antigen in a single bee sting.

As may well be imagined, **antihistamines** offer some relief in allergies; it is usually incomplete, however, since other inflammatory agents are also released from the mast cells. In severe cases, the anti-inflammatory powers of large doses of **cortisol** are employed.

Given the inappropriateness of most immediate hypersensitivity responses, why should such a system have evolved? The normal physiological function of the IgE–mast-cell–eosinophil pathways is to repel invasion by parasitic worms; the eosinphils in such cases serve as the major killer cells against the worms.

Autoimmune Disease

Given the huge diversity of recognition sites for antigens on lymphocytes, why does the immune system not attack the body's cells? Put in a different way, how does the immune system discriminate between self and nonself antigens? In some cases, the self molecules are located deep within cells and tissues and never make contact with lymphocytes. In other cases, the self molecules are not associated with HLA antigens and so fail to stimulate lymphocytes. These two cases offer no problem, but many other self antigens are accessible to lymphocytes and do associate with HLA antigens. It is thought that during in utero and early postnatal life, the clones of lymphocytes that would recognize these self molecules are either destroyed outright or survive but are repressed by suppressor T cells. How these events occur is not understood, although there is general agreement that they occur in the thymus.

Unfortunately, the body does, all too often, produce antibodies or sensitized T cells against its own tissues, the result being cell damage or alteration of function. A growing number of human diseases are being recognized as **autoimmune** in origin.

There are multiple potential causes for the body's failure to recognize its own cells: (1) normal antigens may be altered by combination with drugs or environmental chemicals; (2) the cell may be infected by a virus whose nucleic acid codes for a new protein (antigen); (3) genetic mutations may yield new antigens; (4) the body may encounter microbes whose antigens are so close in structure to certain self antigens that the antibodies or sensitized lymphocytes produced against these antigens cross-react with the self antigens; and (5) suppressor T cells may normally prevent the production of autoantibodies or activated. cytotoxic T cells, and a deficiency of these suppressor T cells could contribute to the development of autoimmunity. This list of possibilities is by no means complete, but whatever the cause, a breakdown in self-recognition results in turning the body's immune mechanisms against its own tissues.

The above description centers on the production of antibodies or sensitized lymphocytes against the body's own cells. However, autoimmune damage may also be brought about in several other quite different ways. An overzealous response (too much generation of complement or release of chemicals from platelets, neutrophils, or sensitized lymphocytes) may cause damage not only to invading foreign cells but to neighboring normal cells or membranes as well. For example, were a circulating antigen-antibody complex to be trapped within capillary membranes, the generation of complement or release of chemicals into the area might cause damage to the adjacent membranes. As might be predicted, the kidney glomeruli, with their large filtering surface, are prime targets for such so-called **immune-complex disease**.

Another important question in the field of autoimmunity concerns the relationship between mother and fetus: Why does the mother's immune system not reject the fetus (half of the fetal antigens are, of course, paternal and therefore foreign to the mother)? No generally accepted explanation is available at present.

Summary

Table 17-6 presents a summary of immune mechanisms in the form of a miniglossary of cells and chemical mediators involved in immune functions. All the material in this table has been covered in this chapter.

TABLE 17-6. Miniglossary of cells and chemical mediators involved in immune functions

Cells

B cells (B lymphocytes). lymphocytes which, upon activation, proliferate and differentiate into antibody-secreting plasma cells

cytotoxic T cells. class of T lymphocytes which, upon activation by specific antigen, directly attack the cell bearing that type of antigen

eosinophils. polymorphonuclear granulocytic leukocytes involved in allergic responses and destruction of parasitic worms

helper T cells. class of T cells which enhance antibody production and cytotoxic T-cell function

killer (K) cells. class of cells (probably lymphocytes) which are brought into action by the presence of antibody bound to antigen on the surface of a foreign cell and which kill the cell directly without prior phagocytosis

lymphocytes. type of leukocyte responsible for specific immune defenses; categorized mainly as B cells and T cells

macrophages. cell type which functions as a phagocyte, processes and presents antigen to lymphocytes, and secretes chemicals involved in inflammation, proliferation of activated lymphocytes, and total-body responses to infection or injury

memory cells. B and T cells produced during an initial infection and which respond rapidly during a subsequent exposure to the same antigen

monocytes. type of leukocyte; leaves the bloodstream and is transformed into a macrophage

natural killer (NK) cells. class of cells (probably lymphocytes) which bind relatively nonspecifically to cells bearing foreign antigens and kill them directly; no prior exposure to the antigen is required

neutrophils. polymorphonuclear granulocytic leukocytes which function as phagocytes and also release chemicals involved in inflammation

plasma cells. cells which are derived from activated B lymphocytes and which secrete antibodies

suppressor T cells. class of T cells which inhibit antibody production and cytotoxic T-cell function

T cells (T lymphocytes). lymphocytes derived from precursors that at one time were in the thymus; see cytotoxic T cells, helper T cells, and suppressor T cells

Chemical Mediators

antibody. specialized protein secreted by plasma cells and capable of combining with the specific antigen which stimulated its production

"blocking" antibodies. antibodies whose production is induced by cancer cells or tissue transplants and which block the killing of these cells by cytotoxic T cells

C_1. The first protein in the classical complement pathway

C_3 convertase. enzyme in the complement pathway which cleaves complement C_3 and initiates the rest of the cascade

chemotaxin. general name given to any chemical mediator which stimulates chemotaxis of neutrophils or other phagocytes

complement. group of plasma proteins which, upon activation, kill microbes directly and facilitate every step of the inflammatory process, including phagocytosis; the classical complement pathway is triggered by antigen-antibody complexes, whereas the alternate pathway operates independently of antibody

endogenous pyrogen (EP). protein secreted by monocytes and macrophages, and which acts in the brain to cause fever; probably the same molecule as LEM and IL-1

histamine. inflammatory mediator secreted mainly by mast cells; acts on microcirculation to cause vasodilation and increased permeability to protein

IgA. class of antibodies secreted by the lining of the body's various "tracts"

IgD. class of antibodies whose function is unknown

IgG. most abundant class of plasma antibodies

IgE. class of antibodies which mediates immediate hypersensitivity ("allergy")

TABLE 17-6. (continued)

Chemical Mediators

IgM. class of antibodies that along with IgG provide the bulk of specific humoral immunity against bacteria and viruses

immunoglobulin (Ig). synonym for "antibody"; the five classes are IgG, IgM, IgA, IgE, and IgD

interferon. family of proteins which nonspecifically inhibit viral replication and also stimulate the activity of cytotoxic T cells, NK cells, and macrophages

interleukin 1 (IL-1). protein secreted by macrophages which stimulates activated B cells to proliferate and helper T cells to secrete interleukin 2; probably the same molecule as LEM and EP

interleukin 2 (IL-2). protein secreted by helper T cells which causes activated T cells to proliferate; its secretion is stimulated by interleukin 1

kinins. peptides split from kininogens in inflamed areas and which facilitate the vascular changes associated with inflammation; may also activate neuronal pain receptors

lactoferrin. protein secreted by neutrophils and which tightly binds iron

leukocyte endogenous mediator (LEM). protein secreted by monocytes and macrophages which induces decreases in plasma zinc and iron, release of acute-phase proteins, and stimulation of granulocyte production; probably the same molecule as EP and IL-1

lymphokines. general term denoting all nonantibody chemical messengers secreted by lymphocytes

membrane attack complex (MAC). group of complement proteins which imbed in the surface of a microbe and kill it

natural antibodies. antibodies to the erythrocyte antigens not present in a person's body; these antibodies are present without prior exposure to the antigen

opsonin. general name given to any chemical mediator which promotes phagocytosis

prostaglandins. mediators produced by many cells, including mast cells, and which facilitate inflammation

SECTION B.
METABOLISM OF FOREIGN CHEMICALS

The body is exposed to a huge number of environmental chemicals, including inorganic nonnutrient elements, naturally occurring fungal and plant toxins, and synthetic chemicals. This last category is by far the largest, since there are now more than 10,000 foreign chemicals being commercially synthesized (over 1 million have been synthesized at one time or another); these are "foreign" in the sense that they are not normally found in nature. These foreign chemicals inevitably find their way into the body, either because they are purposely administered, as drugs (medical or "recreational"), or simply because they are in the air, water, and food we use.

As described in section A of this chapter, foreign materials can induce inflammation and specific immune responses. However, these defenses are directed mainly against foreign cells, and although noncellular foreign chemicals can also elicit certain of them (as in allergy, for example), such immune responses do not constitute the major defense mechanisms against most foreign chemicals. Rather, molecular alteration (**biotransformation**) and **excretion** constitute the primary mechanisms.

A central focus for the body's handling of any foreign chemical is those factors which determine the effective concentration of the chemical at its sites of action (Fig. 17-24). First, the chemical must gain entry to the body through the gastrointestinal tract, lungs, or skin (or placenta in the case of a fetus). Accordingly, its ability to move across these barriers will have an important influence on its blood concentration. But as Fig. 17-24 illustrates, the rate of entry into the body is only one of many factors determining the concentration of the chemical at its site of action. Once in the blood, the chemical may become bound reversibly to plasma proteins or to erythrocytes; this lowers its free concentration and, thereby, its ability to alter cell function. It may accumulate in storage depots (for example, DDT in fat tissue) or it may undergo biotransformation. The metabolites resulting from

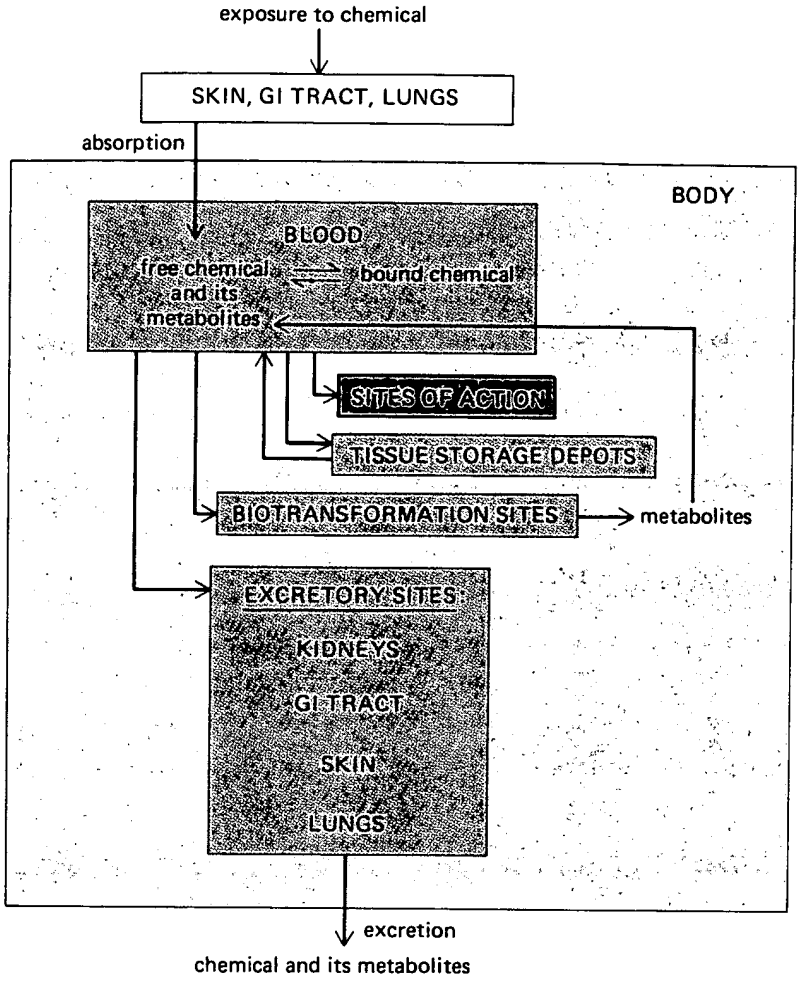


FIGURE 17-24. Metabolic pathways for foreign chemicals.

these latter enzyme-mediated reactions themselves enter the blood and are subject to the same fates as the parent molecules. Finally, the foreign chemical and its metabolites may be eliminated from the body in the urine, expired air, skin secretions, or feces (having entered the feces by biliary secretion).

Absorption

In practice, most organic molecules move through the lining of some portion of the gastrointestinal tract fairly readily, either by simple diffusion or by carrier-mediated transport. This should not be surprising, since the gastrointestinal tract evolved to favor absorption of the wide variety of nutrient molecules in the environment; the nonnutrient chemicals are the beneficiaries of these relatively nondiscriminating transport mechanisms.

The lung alveoli are highly permeable to most organic chemicals and therefore offer an easy entrance route for airborne chemicals. They are also an important entry site for airborne metals, which generally penetrate the gastrointestinal tract very poorly. One important aspect of absorption through the lungs is that the liver does not get first crack at the chemical (as it does when entry is via the gastrointestinal tract); by the same token, any chemical

which is toxic to the liver is not as dangerous when it enters via the lungs.

Lipid solubility is all-important for entry through the skin, so that this route is of little importance for charged molecules but can be used by oils, steroids, and other lipids.

The penetration of the placental membranes by foreign chemicals is one of the most important fields in toxicology, since the effects of environmental agents on the fetus during critical periods of development may be quite marked and, in many cases, irreversible. Diffusion is an important mechanism for lipid-soluble substances, and carrier-mediated systems (which evolved for the carriage of endogenous nutrients) may be usurped by foreign chemcials to gain entry into the fetus.

Storage Sites

The major storage sites for foreign chemicals are cell proteins, bone, and fat. The chemical bound to cell or bone proteins or dissolved in the fat is in equilibrium with the free chemical in the blood, so that an increase in blood concentration causes more movement into storage (up to the point of saturation). Conversely, as the chemical is eliminated from the body and its blood concentration falls, movement occurs out of storage sites.

These storage sites obviously are a source of protection, but it sometimes happens that the storage sites accumulate so much chemical that they become damaged themselves.

Excretion

To appear in the urine, a chemical must either be filtered through the glomerulus or secreted across the tubular epithelium (Chap. 13). Glomerular filtration is, as emphasized in Chap. 13, a bulk-flow process so that all low-molecular-weight substances in plasma undergo filtration; accordingly, there is considerable filtration of most environmental chemicals except for those which are bound to plasma proteins or erythrocytes. (Note that such binding is, therefore, a mixed blessing in that it reduces toxicity but impedes excretion). In contrast, tubular secretion is by discrete transport processes, and many environmental chemicals (penicillin is a good example) utilize the mediated-transport systems available for naturally occurring substances.

Once in the tubular lumen, either via filtration or tubular secretion, the foreign chemical may still not be excreted, for it may be reabsorbed back across the tubular epithelium into the blood. This is a major problem, since so many foreign chemicals are highly lipid-soluble; as the filtered fluid moves along the renal tubules, these molecules passively diffuse along with reabsorbed water through the tubular epithelium and back into the blood. The net result is that little is excreted in the urine, and the chemical is retained in the body. If these chemicals could be transformed into more polar (and, therefore, less lipid-soluble) molecules, their passive reabsorption from the tubule would be retarded and they would be excreted more readily. This type of transformation is precisely what occurs in the liver, as described in the next section.

An analogous problem exists for those foreign molecules (and trace metals) secreted in the bile. Many of these substances, having reached the lumen of the small intestine, are absorbed back into the blood, thereby escaping excretion in the feces. This cyclic enterohepatic circulation was described in Chap. 14.

Biotransformation

The metabolic alteration of foreign molecules occurs mainly in the liver (but to some extent also in kidney, skin, placenta, and other organs). A large number of distinct enzymes and pathways are involved, but the common denominator of most of them is that they transform chemicals into more polar, less lipid-soluble substances. One consequence of this transformation is that the chemical may be rendered less toxic, but this is not always so. The second, more important, consequence is that its tubular reabsorption is diminished and urinary excretion facilitated. Similarly, for substances

handled by biliary excretion, gut absorption of the metabolite is less likely so that fecal excretion is also enhanced.

The hepatic enzymes which perform these transformations are called the **microsomal enzyme system (MES)** and are located mainly in the smooth endoplasmic reticulum. One of the most important facts about this enzyme system is that it is easily inducible, i.e., the number of these enzymes can be greatly increased by exposure to a chemical which acts as a substrate for the system.

However, all is not really so rosy, for the hepatic biotransformation mechanisms vividly demonstrate how an adaptive response may, under some circumstances, turn out to be maladaptive. These enzymes all too frequently "toxify" rather than "detoxify" a drug or pollutant; in fact many foreign chemicals are quite nontoxic until the liver enzymes biotransform them. Of particular importance is the likelihood that many chemicals which cause cancer do so only after biotransformation. For example, a major component of cigarette smoke and charcoal-broiled foods is transformed by the MES into a carcinogenic compound.

The MES can also cause problems in another way, because they evolved primarily not to defend against foreign chemicals (which were much less prevalent during our evolution) but rather to metabolize endogenous substrates, particularly steroids and other lipid-soluble molecules. Therefore, their induction by a drug or pollutant increases metabolism not only of that drug or pollutant, but of the endogenous substrates as well. The result is a decreased concentration in the body of that normal substrate.

Another fact of great importance concerning the microsomal enzyme system is that just as certain chemicals induce it, others inhibit it. The presence of such chemicals in the environment could have deleterious effects on the system's capacity to protect against those chemicals it transforms. (Just to illustrate how complex this picture can be, note that any chemical which inhibits the microsomal enzyme system may actually confer protection against those other chemicals which must undergo transformation in order to become toxic.)

Alcohol: An example

Ethyl alcohol offers an excellent example of the role biotransformation plays in determining a substance's toxicity and its influence on the body's responses to other chemicals. The overuse of alcohol is associated with liver damage, and for many years it was thought that this damage was due to the malnutrition so frequently accompanying alcoholism. It is now clear that, although severe malnutrition may play some role, the toxic damage to the liver is caused mainly by the metabolites of alcohol, produced by the liver cells, themselves.

Alcohol is initially broken down by liver cells to hydrogen and acetaldehyde, and these seem to be the major culprits. Acetaldehyde damages mitochondria. The excess hydrogen exerts its damaging effect more subtly by influencing metabolism so as to cause the accumulation of fat in liver cells, which damages them. (We have restricted our discussion to the toxic effects of hydrogen and acetaldehyde on liver cells; other organs and tissues are also damaged by them in a variety of ways.)

The metabolism of alcohol not only damages the liver cells but leads to marked changes in the metabolism of other drugs and foreign chemicals, all explainable by the fact that these other agents share certain of alcohol's metabolic pathways. First, when alcohol is taken at the same time as another drug, such as a barbiturate, enhanced effects of the two drugs are observed. This is because the alcohol and barbiturate compete for the same hepatic microsomal enzyme system, resulting in a decreased rate of catabolism of both (and, therefore, increased blood concentrations).

The situation just described was for simultaneous administration of the two drugs and is independent of whether the individual is a chronic overuser of alcohol or not. Let us look now at the long-term effects of chronic overuse. Alcohol is a powerful inducer of the microsomal enzyme system, so that its chronic presence causes an increase in the system's activity. The result is an increase in the rate of catabolism of other chemicals, such as barbiturates, which share the enzymes. Thus, the chronic use of alcohol decreases the potency of any

given dose of barbiturate by causing its blood concentration to fall more rapidly.

Putting these last two paragraphs together, one can see that the effect of alcohol on the blood concentrations of other chemicals depends both on whether the two drugs are taken simultaneously and whether the person is a chronic overuser of alcohol. But the situation is even more complex, for if the person has suffered serious liver damage due to chronic alcoholism, then the microsomal enzyme system, instead of being induced, may be inadequate simply because the cells have been damaged. At this stage, then, the metabolism of the other agents will be diminished and their effects will be exaggerated.

Finally, we have presented the chronic effects of alcohol only in terms of the effects on the metabolism of other chemicals. It should be clear, however, that similar effects are exerted on the metabolism of alcohol itself. Thus, chronic overuse, before significant damage has occurred, results in increased catabolism (because of the induction of the microsomal enzymes), and this accounts for much of the "tolerance" to alcohol, i.e., the fact that increasing doses must be taken to achieve a given magnitude of effect. Once severe damage has occurred the decline in rate of catabolism may lead to increased sensitivity to a given dose, just the opposite of tolerance.

Inorganic Elements

Thus far our discussion of environmental chemicals has dealt mainly with organic molecules. Many potentially dangerous substances are not organic chemicals but are inorganic elements (like mercury and lead) present in excess in the environment because of human activity. As is true for organic drugs and pollutants, the concentrations of inorganic elements at their sites of action depend on rates of entry, excretion, and storage. The gastrointestinal tract offers an important first line of defense since absorption of them is usually quite limited. In contrast, airborne inorganic elements gain entry to the blood more readily through the lungs. Little is known of the mechanisms by which the kidneys and liver handle potentially harmful trace elements; specifically, it is not known whether adaptive increases in excretion are induced by exposure to the element.

SECTION C.
RESISTANCE TO STRESS

Much of this book has been concerned with the body's response to stress in its broadest meaning of an environmental change which must be adapted to if health and life are to be maintained. Thus, any change in external temperature, water intake, etc., sets into motion mechanisms designed to prevent a significant change in some physiological variable. In this section, however, we describe the basic stereotyped response to **stress** in the more limited sense of noxious or potentially noxious stimuli. These comprise an immense number of situations, including physical trauma, prolonged heavy exercise, infection, shock, decreased oxygen supply, prolonged exposure to cold, pain, fright, and other emotional stresses. It is obvious that the overall response to cold exposure is very different from that to infection, but in one respect the response to all these situations is the same: Invariably, secretion of **cortisol** is increased; indeed, the term "stress" has come to mean to physiologists any event which elicits increased cortisol secretion (by the **adrenal cortex**). Also, **sympathetic nervous activity** is usually increased.

Historically, activation of the sympathetic nervous system was the first overall response to stress to be recognized and was labeled the **fight-or-flight**

response. Only later did further work clearly establish the contribution of the adrenal cortical response. The increased cortisol secretion is mediated entirely by the hypothalamus–anterior-pituitary system described in Chap. 9. As illustrated in Fig. 17-25, afferent input to the hypothalamus induces secretion of **corticotropin releasing hormone,** which is carried by the hypothalamo-pituitary portal vessels to the anterior pituitary and stimulates **ACTH** release. The ACTH, in turn, circulates to the adrenal and stimulates cortisol release. As described in Chap. 9, the hypothalamus receives input from virtually all areas of the brain and receptors of the body, and the pathway involved in any given situation depends upon the nature of the stress, e.g., pathways from other brain centers mediate the response to emotional stress. The destination is always the same, namely, synaptic connection with the hypothalamic neurons which secrete corticotropin releasing hormone. These same pathways also converge on the brain areas which control sympathetic nervous activity (including release of **epinephrine** from the **adrenal medulla**).

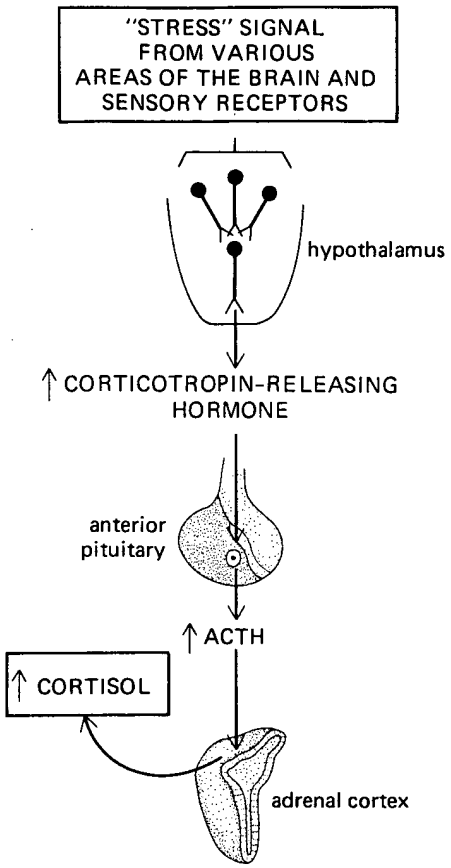


FIGURE 17-25. Pathway by which stressful stimuli elicit increased cortisol secretion.

Functions of Cortisol in Stress

The effects of increased cortisol on organic metabolism were described in Chap. 15. To reiterate, cortisol (1) stimulates protein catabolism; (2) stimulates liver uptake of amino acids and their conversion to glucose (gluconeogenesis); and (3) inhibits glucose uptake and oxidation by many body cells ("insulin antagonism") but not by the brain. These effects are ideally suited to meet a stressful situation. First, an animal faced with a potential threat is usually forced to forego eating, and these metabolic changes are essential for survival during fasting. Second, the amino acids liberated by catabolism of body protein stores not only provide energy, via gluconeogenesis, but also constitute a potential source of amino acids for tissue repair should injury occur.

A few of the many medically important implications of these cortisol-induced metabolic effects associated with stress are as follows: (1) Any patient ill or subject to surgery catabolizes considerable quantities of body protein; (2) a diabetic who suffers an infection requires much more insulin than usual; and (3) a child subject to severe stress of any kind manifests retarded growth. The explanations for these phenomena should be evident.

Cortisol has important effects other than those on organic metabolism. One of the most important is that of enhancing **vascular reactivity**. A patient lacking cortisol faced with even a moderate stress may develop hypotension and die if untreated. This is due primarily to a marked decrease in total peripheral resistance. For unknown reasons, stress

would induce widespread arteriolar dilation, despite massive sympathetic nervous system discharge, if large amounts of cortisol were not present. A large part of its counteracting effect is ascribable to the fact that moderate amounts of cortisol permit norepinephrine to induce vasoconstriction, but this can be only part of the story since considerably larger amounts of cortisol are required to prevent stress-induced hypotension completely. In other words, the normal cardiovascular response to stress requires *increased* cortisol secretion, not just permissive quantities.

Thus far we have presented the adaptive value of the stress-induced cortisol increase mainly in terms of its role in preparing the body physically for fight or flight, and there is no doubt that cortisol does function importantly in this way. However, in recent years, it has become apparent that cortisol may have other important functions. Table 17-7 is a partial list of the large variety of psychosocial situations demonstrated to be associated with increased cortisol secretion. Common denominators of many of them are novelty and challenge. Of great interest, therefore, are recent experiments which

TABLE 17-7. Psychosocial situations shown to be associated with increased plasma concentration or urinary excretion of adrenal cortical steroids

Experimental animals

1. Any "first experience" characterized by novelty, uncertainty, or unpredictability
2. Conditioned emotional responses; anticipation of something previously experienced as unpleasant
3. Involvement in situations in which the animal must master a difficult task in order to avoid or forestall aversive stimuli (the animal must really be "trying")
4. Situations in which long-standing rules are suddenly changed so that previous behavior is no longer effective in achieving a goal
5. Socially subordinate animals (dominant animals have decreased cortisol)
6. Crowding (increased social interactions)
7. Fighting or merely observing other animals fighting

Human beings

1. Normal persons
   - *a.* Acute situations
     - (1) Aircraft flight
     - (2) Awaiting surgical operation
     - (3) Final exams (college students)
     - (4) Novel situations
     - (5) Competitive athletics
     - (6) Anticipation of exposure to cold
     - (7) Workdays, compared to weekends
     - (8) Many job experiences
   - *b.* Chronic life situations
     - (1) Predictable personality-behavior profile: aggressive, ambitious, time-urgency
     - (2) Discrepancy between levels of aspiration and achievement
   - *c.* Experimental techniques
     - (1) "Stress" or "shame" interview
     - (2) Many motion pictures
2. Psychiatric patients
   - *a.* Acute anxiety
   - *b.* Depression, but only when patient is aware of and involved in a struggle with it

suggest that cortisol affects memory in experimental animals. Even more striking, ACTH (independent of its stimulation of cortisol secretion) is one of the peptides which facilitates learning and memory (Chap. 20). Thus, it may well be that the rise in ACTH secretion induced by psychosocial stress helps one to cope with the stress by facilitating the learning of appropriate responses.

Cortisol's pharmacological effects and disease

There are several situations in which adrenal corticosteroid levels in human beings become abnormally elevated. Patients with excessively hyperactive adrenals (there are several causes of this disease) represent one such situation, but the common occurrence is that of steroid administration for medical purposes. When corticosteroids are present in very high concentration, the previously described effects on organic metabolism are all magnified, but in addition there may appear one or more new effects, collectively known as the **pharmacological effects** of cortisol and closely related steroids. The most obvious is a profound reduction in the inflammatory response to injury or infection (indeed, reducing the inflammatory response in allergy, arthritis, other diseases, and transplantation rejection is the major reason for administering the cortisol to patients). Large amounts of cortisol inhibit almost every step of inflammation (vasodilation, increased vascular permeability, phagocytosis), destroy lymphocytes in lymphoid tissues, and decrease antibody production. Unfortunately, such therapy also decreases the ability of the person to resist infections. In addition, large amounts of cortisol may accelerate development of hypertension, atherosclerosis, and gastric ulcers, and may interfere with normal menstrual cycles.

As emphasized above, these pharmacological effects are known to be elicited when cortisol levels are extremely elevated. An unsettled question of great importance is whether long-standing lesser elevations of cortisol may do the same thing, albeit more slowly and less perceptibly. Put in a different way, do the psychosocial stresses, noise, etc., of everyday life contribute to disease production via increased cortisol?

Functions of the Sympathetic Nervous System in Stress

A list of the major effects of increased general sympathetic activity, including secretion of adrenal medullary hormones, almost constitutes a guide on how to meet emergencies. Since all these actions have been discussed in other sections of the book, they are listed here with little or no comment:

1. Increased hepatic and muscle glycogenolysis (provides a quick source of glucose)
2. Increased breakdown of adipose tissue triacylglycerol (provides a supply of glycerol for gluconeogenesis and of fatty acids for oxidation)
3. Decreased fatigue of skeletal muscle
4. Increased cardiac output secondary to increased cardiac contractility and heart rate
5. Shunting of blood from viscera to skeletal muscles by means of vasoconstriction in the former beds and vasodilation in the latter
6. Increased ventilation
7. Increased coagulability of blood

The adaptive value of these responses in a fight-or-flight situation is obvious. But what purpose do they serve in the psychosocial stresses so common to modern life when neither fight nor flight is appropriate? As for cortisol, a question yet to be answered is whether certain of these effects, if prolonged, might not enhance the development of certain diseases, particularly atherosclerosis and hypertension. For example, one can easily imagine the increased blood lipid concentration and cardiac work contributing to the former disease. Considerable work remains to be done to evaluate such possibilities.

Other Hormones Released During Stress

Other hormones which are usually released during many kinds of stress are aldosterone, vasopressin (ADH), growth hormone, and glucagon (insulin is usually decreased). The increases in vasopressin and aldosterone ensure the retention of water and

sodium within the body, an important adaptation in the face of potential losses by hemorrhage or sweating (vasopressin may also influence learning). As described in Chap. 15, the overall effects of the changes in growth hormone, glucagon, and insulin are, like those of cortisol and epinephrine, to mobilize energy stores.

This list of hormones whose secretion rates are altered by stress is by no means complete. It is likely that the secretion of almost every known hormone may be influenced by stress. For example, prolactin and thyroid hormone are often increased, whereas the pituitary gonadotropins (LH and FSH) and the sex steroids (testosterone or estrogen) are decreased. The adaptive significance of many of these changes is unclear.

The secretion of endorphin and B-lipotropin is also increased during stress. As described in Chap. 9, these substances are secreted from the anterior pituitary along with ACTH. Endorphin is a potent endogenous opiate, and its possible role in mediating analgesia and mood alterations in stress is a subject of great interest. Moreover, endorphin has many other effects as well; for example, it decreases appetite.

Finally, we would like to raise once again the potential role of the EP/LEM/IL-1 hormone(s) secreted by stimulated macrophages. To the extent that any particular stress actually causes significant tissue damage, it will trigger release of these proteins, which exert the far-reaching protective effects described earlier. A question for future study is whether psychological stresses or physical stresses that cause little, if any, tissue damage can also trigger this system, perhaps via hormonal input to the macrophages.

CELLULAR AND MOLECULAR IMMUNOLOGY

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CHAPTER SIX

ANTIGEN PRESENTATION AND T CELL ANTIGEN RECOGNITION

The induction of humoral and cell-mediated immune responses to protein antigens requires the recognition of the antigens by helper T cells. The reason for this is that helper T cells are necessary for stimulating B lymphocyte growth and differentiation and for activating the effector cells of cell-mediated immunity, namely macrophages and cytolytic T lymphocytes (CTLs). Before we discuss the activation of T cells by protein antigens and the functions of various T cell subsets in subsequent chapters, we need to understand the structures of antigens that are recognized by T cells. The concept that major histocompatibility complex (MHC)-encoded molecules play an important role in antigen recognition by helper cells and CTLs was introduced in Chapter 5. This chapter describes the formation of complexes of foreign antigens and MHC molecules, which are the ligands for which T cell antigen receptors are specific, and the physiologic significance of this unusual specificity.

CHARACTERISTICS OF ANTIGEN RECOGNITION BY T LYMPHOCYTES

It is now known that CD4+ T lymphocytes, most of which are helper cells, recognize peptides that are bound to class II MHC molecules on the surfaces of other, non-T cells. Class II-associated peptides are usually derived from extracellular microbes and soluble protein antigens. Furthermore, CD8+ T cells, most of which are CTLs, recognize peptide fragments bound to class I MHC molecules on cells that are targets of the lytic action of CTLs. These class I-associated peptides are generally derived from endogenously synthesized proteins, such as viral antigens. The elucidation of these principles was one of the most impressive achievements in immunology in the 1980s. Our current understanding of T cell antigen recognition is the culmination of a vast amount of work beginning with studies on the physicochemical forms of antigens that stimulated cell-mediated immunity. These studies led to the discovery that cells other than T lymphocytes play an obligatory role in T cell activation by foreign antigens and later to the elucidation of the function of MHC molecules in T cell antigen recognition.

Physicochemical Forms of Antigens Recognized by T Lymphocytes

The realization that humoral and cell-mediated immunity are mediated by different classes of lymphocytes, i.e., B and T cells, respectively, led many investigators to examine the properties of the antigens that stimulated these two types of immune responses. Such studies established a fundamental concept, namely that *T lymphocytes recognize different forms of antigens from B lymphocytes and secreted immunoglobulin* (Ig).

1. T lymphocytes recognize only protein antigens, whereas B cells can specifically recognize proteins, nucleic acids, polysaccharides, lipids, and small chemicals. Some T cells are specific for chemically reactive forms of haptens such as dinitrophenol. In these situations, it is likely that the haptens bind to cell surface proteins, including MHC molecules, and these hapten-protein conjugates are recognized by T cells. As we shall see later, the reason why T cells respond only to protein antigens is that only fragments of proteins can form stable complexes with MHC molecules.

2. B cells specific for protein antigens may recognize conformational determinants that exist when proteins are in their native tertiary (folded) configuration or determinants that are exposed by denaturation or proteolysis. In contrast, T cells recognize only linear determinants defined predominantly by primary amino acid sequences. Thus, when an animal is immunized with a native protein, the antibodies it produces will react only with the native protein. In contrast, the antigen-specific T cells which are stimulated by immunization with the native protein will respond to denatured or even proteolytically digested forms of that protein (Table 6-1). Consistent with this difference in the nature of antigenic determinants for T and

TABLE 6-1. Qualitative Differences in Antigen Recognition by T and B Lymphocytes

		Secondary Immune Response	
Immunizing Antigen	**Secondary Antigen Exposure**	***B Cell-Mediated (Antibody Production)***	***T Cell-Mediated (Delayed Type) Hypersensitivity***
Native protein	Native protein	+	+
Denatured protein	Native protein	−	+
Native protein	Denatured protein	−	+
Denatured protein	Denatured protein	+	+

Antigen recognition by T and B lymphocytes is qualitatively different. In an immunized animal, B cells are specific for conformational determinants of the immunogen and, therefore, distinguish between native and denatured protein antigens. T cells, however, do not distinguish between native and denatured protein antigens because T cells recognize non-conformational linear epitopes.

B cell recognition is the finding that T cell responses to a soluble antigen cannot be inhibited using antibodies specific for conformational determinants of that antigen, whereas antigen recognition by B cells can be competitively inhibited by such antibodies.

Role of Accessory Cells in T Cell Responses to Antigens

The second important characteristic of antigen recognition by T lymphocytes is that *T cells recognize and respond to foreign protein antigens only when the antigen is attached to the surfaces of other cells,* whereas B cells and secreted antibodies bind soluble antigens in the circulation or in the aqueous phase. Thus, CTLs recognize antigens bound to the surface of target cells and kill these targets. The activation of helper T cells by foreign antigens requires the participation of cells other than T lymphocytes; these are called **accessory cells.** These accessory cells serve two principal functions in helper T cell stimulation:

1. Accessory cells display fragments of foreign protein antigens on their surfaces in a form that can be specifically recognized by T cell antigen receptors. This phenomenon is called **antigen presentation,** and the cell populations capable of performing this function are **antigen-presenting cells** (APCs). (The term APCs is used for accessory cells that present antigens to helper T lymphocytes. Since CTLs also recognize foreign antigens bound to the surfaces of their target cells, all such target cells may be conceptually included among APCs. Conventionally, however, cells that are recognized and lysed by CTLs are called **target cells,** not APCs.)
2. Accessory cells provide stimuli to the T cell, beyond those initiated by ligand binding to the T cell antigen receptor, which are required for physiologic activation. These stimuli, referred to as **costimulator activities,** are incompletely characterized. They may be provided by membrane-bound or secreted products of accessory cells.

The antigen-presenting functions of accessory cells are described in more detail later in this chapter, and their costimulator functions are discussed in Chapter 7.

The importance of APCs in initiating T cell-dependent immune responses was first suggested in the 1950s by the demonstration that radioactively or fluorescently labeled antigens injected into animals were found in mononuclear phagocytes or follicular dendritic cells and not in lymphocytes. Later studies showed that an antigen that was bound to macrophages *in vitro* and then injected into mice was up to 1000 times more immunogenic on a molar basis than the same antigen administered by itself, in a cell-free form. The explanation for this finding is that T cells respond only to antigen associated with macrophages or other APCs, and only a small fraction of an injected soluble antigen ends up in this immunogenic cell-associated form.

The obligatory role of accessory cells in lymphocyte activation was formally established when techniques for stimulating immune responses *in vitro* were developed. For example, T cells isolated from the blood, spleen, or lymph nodes of individuals immunized with a protein antigen can be restimulated in tissue culture by that antigen. Stimulation may be measured by assaying the production of cytokines by the T cells or by the proliferation of the T cells. When contaminating macrophages and dendritic cells are removed from the cultures, the purified T lymphocytes no longer respond to antigen, and responsiveness can be restored by adding back the macrophages or dendritic cells. Such experimental approaches provide the basis for defining the accessory functions of various cell types in T lymphocyte activation. The importance of accessory cells in immune responses *in vivo* is suggested by the observation that **adjuvants** often need to be administered in addition to antigen in order to elicit an immune response to the antigen. These adjuvants are usually insoluble or undegradable substances that promote nonspecific inflammation, with recruitment of mononuclear phagocytes at the site of immunization.

The Phenomenon of MHC-Restricted Antigen Recognition by T Lymphocytes

The critical advance in our understanding of antigen recognition by helper T cells and CTLs was the discovery of the phenomenon of **self MHC restriction** in the 1970s. *MHC restriction is the requirement that an APC must express MHC molecules that the T cell recognizes as self in order for the T cell to recognize and respond to a foreign protein antigen presented by that APC.* The MHC molecules that T cells recognize as self are those that the T cells encountered during their maturation from precursors in the thymus. (The process of T cell maturation is discussed in much more detail in Chapter 8.) "Self MHC" refers not to MHC molecules expressed by the T cells themselves but to MHC molecules on the APCs or target cells. Normally, because T cells and APCs develop in the same individual, they are syngeneic and all the MHC molecules on the APCs are seen as self MHC by all the T cells in that individual. In experimental systems, T cells respond to antigens presented by a particular APC if the two cell types are at least partly syngeneic, i.e., if they are derived from individuals or inbred strains that share one or more MHC alleles. In this situation the APCs express MHC molecules that the T cells encountered and learned to see as self during their maturation.

This phenomenon of self MHC restriction was discovered when T cells from one inbred strain of animal were mixed with APCs from different inbred strains and T cell responses were assayed. Three sets of ex-

periments established MHC restriction of antigen recognition by helper T cells and CTLs:

1. T cells from an antigen-primed guinea pig of one inbred strain proliferate in response to antigen *in vitro* only if macrophages from the same strain are present. These proliferating T cells are mostly helper cells. Subsequent analyses using inbred and congenic strains of mice revealed that in order to present antigens to helper T cells, the APCs have to express class II MHC molecules that are seen as self by the T cells (Fig. 6-1). Such experiments, and others using purified and monoclonal T cell populations from mice and humans, have established that *antigen recognition by helper T cells is class II MHC-restricted.*

2. *In vivo* experiments with inbred mice utilizing

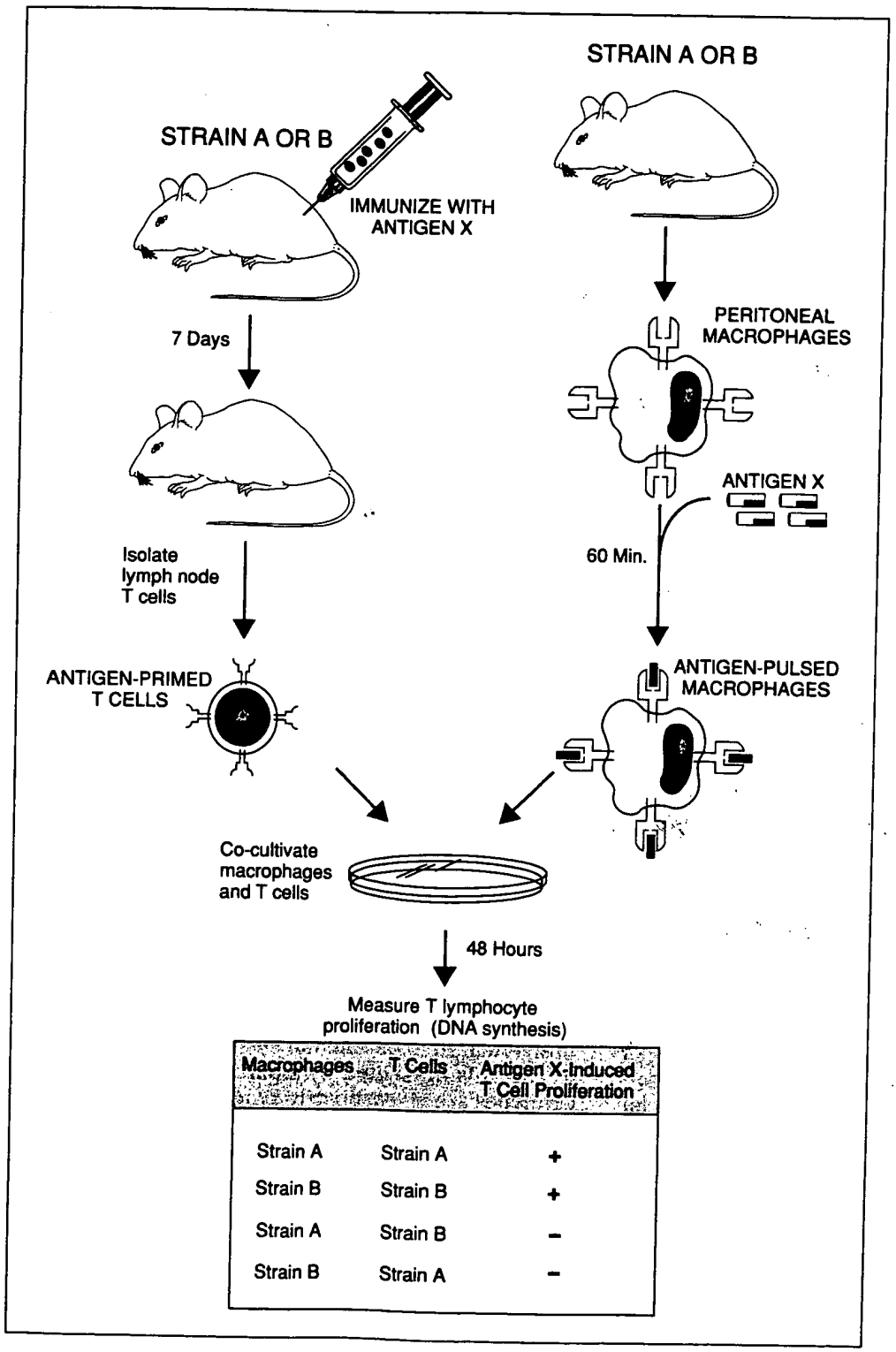
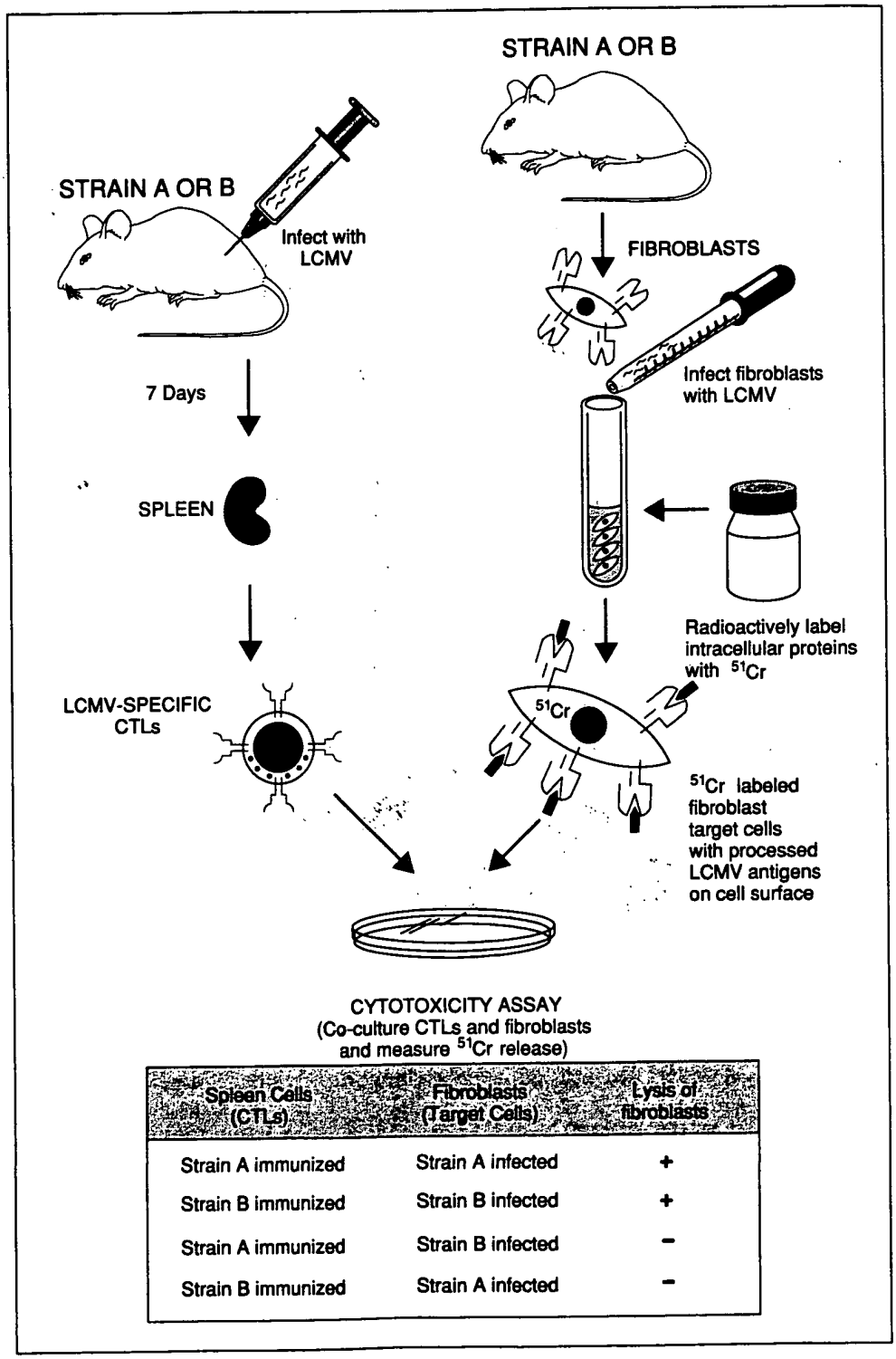


FIGURE 6-1. MHC restriction of proliferating (helper) T lymphocytes. *T cells from a strain A or strain B mouse primed with an antigen X proliferate in response to that antigen only in the presence of strain A or B macrophages (or other antigen-presenting cells [APCs]), respectively. In the experiment depicted, the T cell populations are devoid of alloreactivity; i.e., strain A T cells do not respond to the foreign MHC molecules of strain B, and vice versa. The T cells also do not proliferate in the absence of antigen. Using congenic and recombinant strains of mice, it can be shown that the macrophages and T cells must come from animals that share class II MHC alleles in order for the antigen-induced T cell proliferation to occur.*

adoptive transfer techniques and *in vitro* studies of antibody production showed that helper T lymphocytes and B cells cooperate to produce an antibody response to a protein antigen only if the B cells express class II MHC molecules that are seen as self by the T cells. This phenomenon is discussed in more detail in Chapter 9. It further supports the conclusion that helper T cells are class II MHC-restricted.

3. Perhaps the clearest demonstration of MHC restriction came from assays of virus-specific CTL-mediated lysis of virally infected target cells in mice and humans. In most of these systems, the virus-infected target cells are lysed only if they express class I MHC molecules that are recognized as self MHC by the T cells (Fig. 6-2). This established that *CTL recognition of viral antigens is class I MHC-restricted.*

These experiments suggest that the MHC gene products involved in T cell antigen recognition must



Spleen Cells (CTLs)	Fibroblasts (Target Cells)	Lysis of fibroblasts
Strain A immunized	Strain A infected	+
Strain B immunized	Strain B infected	+
Strain A immunized	Strain B infected	-
Strain B immunized	Strain A infected	-

FIGURE 6-2. MHC restriction of cytolytic T lymphocytes (CTLs). *Virus-specific CTLs from a strain A or strain B mouse lyse only syngeneic target cells infected with the specific virus. The CTLs do not lyse uninfected targets and are not alloreactive. Further analysis has shown that the CTLs and target cells must come from animals that share class I MHC alleles in order for the target cell to present viral antigens to the CTLs. LCMV, lymphocytic choriomeningitis virus.*

TABLE 6-2. Major Histocompatibility Complex (MHC) Restriction of T Cell Responses to Antigen Depends on MHC Molecules on Antigen-Presenting Cells (APCs)

Monoclonal T Cell Population from (A × B)F1 Mouse, Specific for Antigen X	T Cell Response to Antigen X plus APC from Strain			
	A	*B*	*(A × B)F1*	*C*
Clone No. 1 (MHC^B-restricted)	−	+	+	−
Clone No. 2 (MHC^A-restricted)	+	−	+	−

MHC restriction of T cell responses to antigen depends on MHC molecules on the APC. Individual T cells can be isolated from an antigen X-immunized (A × B)F1 mouse, which has both strain A and strain B MHC genes. These T cells are then propagated *in vitro*, giving rise to multiple identical progeny (monoclonal T cell populations). Recognition of antigen X by progeny of T cell clone number 1 depends on the presence of APCs from strain B, whereas T cell clone number 2 requires APCs from strain A. Both T cell clones number 1 and number 2 respond to antigen X in the presence of APCs from an (A × B)F1 mouse. Neither T cell clone will respond to antigen X alone or in the presence of APCs from a third unrelated strain (C), which has no shared MHC genes with A or B. This pattern of responses indicates that the MHC restricting element, i.e., the MHC protein which the T cell recognizes as self, must be expressed by the APC.

be expressed on the APC or on the target for CTL-mediated lysis. Several experiments have proved that this is indeed the case and that the necessary MHC molecules need not be present on the T cells themselves.

1. A monoclonal antigen-specific T cell restricted by MHC of type A responds to that antigen in the presence of APCs that express the type A MHC allele (Table 6-2). Among antigen-specific T cells derived from an (A × B)F1 individual, some of the cells are MHC^A-restricted, others are MHC^B-restricted, and a smaller subset may be restricted by hybrid MHC molecules expressed only in (A × B)F1 mice. The key point is that even though the MHC^A-restricted T cells are from an (A × B) F1 mouse, they only respond to APCs which express MHC^A (either alone or with MHC^B).

2. The response of an MHC^A-restricted T cell to an antigen is blocked by antibodies specific for type A MHC molecules. The lytic activity of $CD8^+$ T cells is inhibited by antibodies against class I MHC molecules, and the activation of $CD4^+$ T cells is inhibited by anti-class II antibodies. Such inhibition is seen only if the antibodies bind to the APC, not if they bind to the T cells (Table 6-3).

3. APCs that do not express restricting MHC molecules fail to stimulate antigen-specific T cells. For instance, in the example mentioned above, MHC^A-restricted T cells do not respond to APCs that are MHC^A-negative. If the genes coding for MHC^A are transfected into the APCs and the cells become MHC^A-positive, they also acquire the ability to stimulate the T cells (Table 6-4).

These experiments led to the fundamental concept that *helper and cytolytic T lymphocytes specific for foreign protein antigens simultaneously recognize two structures, the foreign antigen and a self MHC molecule, both of which are present on the surface of the APC or target cell* (Fig. 6-3). The experiments described above suggested that helper T cells and CTLs are ex-

TABLE 6-3. Inhibition of MHC-Restricted Antigen Presentation by Anti-MHC Antibodies

	T Cells from Immunized Mice	Macrophages	Antibody in Culture	T Cell Proliferation
A	I-A restricted helper T cell	Normal	None	−
	I-A restricted helper T cell	Ag-pulsed	None	+
	I-A restricted helper T cell	Ag-pulsed	anti-I-A	−
	I-A restricted helper T cell	Ag-pulsed	anti-I-E	+
	I-A restricted helper T cell	Ag-pulsed	anti-K/D	+
B	I-A restricted helper T cell + anti-I-A (prebound)	Ag-pulsed	None	+
	I-A restricted helper T cell	Ag-pulsed + anti-I-A (prebound)	None	−

A helper T cell population specific for I-A-associated antigen proliferates in response to the antigen presented by I-A expressing APCs. The response is blocked by anti-I-A antibody (in part *A* of table), but only if the antibody is allowed to bind to the APCs before adding the T cells (part *B*). Therefore, T cells recognize self MHC-associated antigens presented by APC.

Abbreviations: MHC, major histocompatibility complex; Ag, antigen; APC, antigen-presenting cell.

TABLE 6-4. Requirement for Class II MHC Expression in Antigen Presentation to CD4+, Antigen-Specific T Cells

APCs	Genes Transfected Into APCs	Surface Class II MHC	Surface Class I MHC	Antigen	Response of Cytochrome c-Specific, I-E^k-Restricted T Cell Line (Cytokine Secretion)
3T3 (murine fibroblast)	None	None	K^k, D^k	Cytochrome c	−
3T3 (murine fibroblast)	Murine class II $E\alpha^k$ and $E\beta^k$	I-E^k	K^k, D^k	None	−
3T3 (murine fibroblast)	Murine class II $E\alpha^k$ and $E\beta^k$	I-E^k	K^k, D^k	Cytochrome c	+

Class II MHC expression is required for antigen presentation to CD4+, antigen-specific T cells. In this experiment, a murine fibroblast cell line, 3T3, derived from an H-2^k mouse, which expresses class I, but not class II, MHC molecules, does not present cytochrome c to a cytochrome c-specific, I-E^k-restricted, T cell hybridoma line. When functional genes encoding the α and β chains of the I-E^k molecule are transfected into 3T3 cells, they become competent at presenting antigen to the T cell line.

Abbreviations: MHC, major histocompatibility complex; APC, antigen-presenting cell.

clusively restricted by class II and class I MHC molecules, respectively. In fact, the class I or class II restriction of T cells correlates more strongly with their expression of CD8 or CD4 than with the functional capabilities of the cells. Thus, *all CD4+ T cells are restricted by class II MHC molecules,* and in fact CD4 itself binds to class II molecules. Most CD4+ cells are helper cells, although CD4+ CTLs (again class II-restricted) have been identified in humans and mice. *Similarly, all CD8+ T cells are class I-restricted,* and the CD8 molecule binds to class I MHC molecules. Most of these cells are CTLs, although some may function as cytokine-producing helper cells. (Some CD8+ T cells suppress immune responses; the specificity of suppressor cells is a controversial issue and is discussed in Chapter 10.) The structures of CD4 and CD8 molecules and their roles in T cell antigen recognition are discussed in Chapter 7.

The molecular basis of MHC-restricted antigen recognition by T cells was elucidated by parallel studies of the structure of the T cell receptor for antigen and of antigen presentation. As we shall see in Chapter 7, T cells express a single antigen receptor that simultaneously interacts with an epitope of a protein antigen that is bound to MHC molecules and with polymorphic residues of MHC molecules. The analysis of antigen presentation being done concurrently has revealed that *foreign protein antigens are presented to T cells as peptides that are non-covalently attached to the peptide-binding clefts of MHC molecules.* As we discuss below, these studies have demonstrated how large protein antigens can physically associate with MHC molecules. Furthermore, we now understand how the vast number of protein antigens that different T cells can recognize are presented by the few (less than 20) allelic MHC molecules expressed in each individual.

MECHANISMS OF ANTIGEN PRESENTATION

The fundamental feature of antigen presentation to MHC-restricted T cells is that foreign antigens form physical complexes with MHC molecules. The ability to generate such complexes is the essential property of all APCs or targets for CTLs. Thus, APCs are capable of converting even large globular proteins to a sufficiently small size and appropriate conformation that can non-covalently attach to the peptide-binding clefts of MHC molecules synthesized by the APCs. The conversion of native proteins to MHC-associated peptide fragments is called **antigen processing.** Foreign antigens that are synthesized outside the APCs, such as bacterial proteins and soluble protein antigens that are administered to individuals, first bind to APCs and are then endocytosed. At this stage, the proteins may be in their native tertiary forms. They are then partly degraded, and fragments derived from the antigens usually bind to the peptide-binding clefts of class II MHC molecules. Complexes of processed antigens and class II MHC molecules are displayed on the surfaces of APCs, where they are recognized by class II MHC-restricted T lymphocytes. Foreign proteins that are synthesized within a cell, such as viral proteins and tumor antigens, are also processed, but they enter a different intracellular compartment from endocytosed antigens. Peptides derived from endogenously synthesized proteins generally associate with class I MHC molecules, although some endogenously synthesized, e.g., viral, proteins may be processed and may become bound to class II molecules. Complexes of peptides and class I MHC molecules are displayed on the cell surface, where they are recognized by class I-restricted T cells.

This portion of the chapter describes the cell types that can function as APCs, the mechanisms of antigen processing, and the characteristics of peptide-MHC association.

Types of Antigen-Presenting Cells

The two requisite properties that allow a cell to function as an APC for class II MHC-restricted helper T lymphocytes are the ability to process endocytosed antigens and the expression of class II MHC gene products. Most mammalian cells appear to be capable of endocytos-

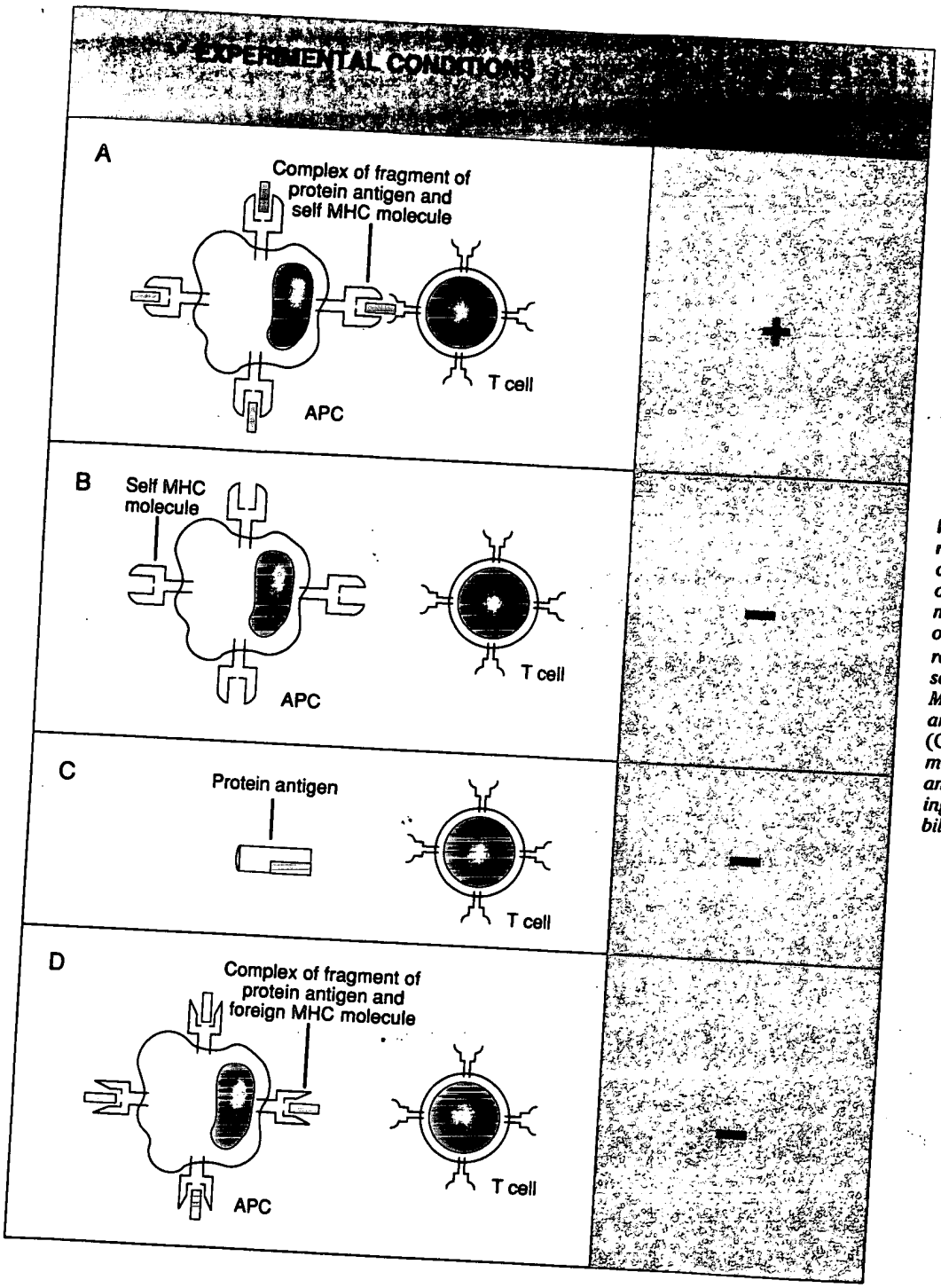


FIGURE 6-3. *Specificity of MHC-restricted T cells. Helper T cells and cytolytic T lymphocytes (CTLs) recognize complexes of self MHC molecules and peptide fragments of foreign antigens* (A). *MHC-restricted T cells do not recognize self MHC molecules alone or self MHC with self peptide* (B), *foreign antigens without MHC molecules* (C), *or complexes of foreign MHC molecules and peptide fragment of antigen* (D). *APC, antigen-presenting cell; MHC, major histocompatibility complex.*

ing and processing protein antigens, so that the critical property that enables a particular cell to function as an APC is the expression of class II MHC molecules.

The best-defined APCs for helper T lymphocytes include: (1) mononuclear phagocytes, (2) B lymphocytes, (3) dendritic cells, (4) Langerhans cells of the skin, and (5) in humans, endothelial cells.

Macrophages and other cells of the **mononuclear phagocyte system** actively phagocytose large particles. Therefore, they probably play an important role in presenting antigens derived from infectious organisms such as bacteria and parasites.

B lymphocytes specific for a protein antigen are very efficient at presenting that antigen to helper T lymphocytes *in vitro* and may serve as APCs *in vivo*, particularly when the concentration of available antigen is low. The reason why antigen-specific B cells are uniquely efficient APCs is that their membrane Ig molecules can bind the antigen with high affinity and,

therefore, at low concentrations. The antigen-presenting function of B cells may be particularly important in helper T cell-dependent antibody production (see Chapter 9).

Dendritic cells of the spleen and lymph nodes are irregularly shaped, nonphagocytic cells making up a small fraction (<1 per cent) of the total cell population of these organs. They are derived from the bone marrow and may be related to the mononuclear phagocytic lineage. Dendritic cells are competent at presenting protein antigens to helper T cells. It is also believed that dendritic cells are important for inducing T cell responses to foreign (allogeneic) MHC molecules in tissue allografts. Consistent with this hypothesis is the observation that dendritic cells are potent stimulators of mixed lymphocyte reactions (see Chapter 16).

Langerhans cells are specialized epidermal cells with a dendritic morphology. They are derived from bone marrow progenitors, express the CD1 marker, and contain an unusual cytoplasmic organelle called the Birbeck granule. Langerhans cells may be related in lineage to the dendritic cells of spleen and lymph nodes. They are the only resident epidermal cells known to be capable of antigen presentation and, therefore, may be important in presenting the antigens responsible for cutaneous contact sensitivity reactions.

In humans, *venular endothelial cells* express class II MHC molecules and may also interact with T cells. This may be particularly important in cell-mediated immune reactions, such as delayed type hypersensitivity reactions in peripheral tissues (see Chapter 12).

In addition to cells that express class II MHC molecules constitutively, many cell types can be induced to express their class II MHC genes by the T lymphocyte-derived cytokine γ interferon (IFN-γ) (Fig. 6-4). Epithelial, glial, mesenchymal, and vascular endothelial cells may acquire antigen-presenting capabilities *in vitro* when they are induced to express class II MHC molecules. The physiologic role of these cell types in immune responses to protein antigens *in vivo* is not well defined. It is likely that immune responses are initiated by APCs that express class II molecules constitutively. This may result in the activation of T cells at the site of antigen exposure, local production of IFN-γ, and increased class II MHC expression on resident APCs as well as on endothelial or mesenchymal cells that normally cannot present antigens. As a result, more cell populations begin to function as APCs, and this may lead to enhanced antigen presen-

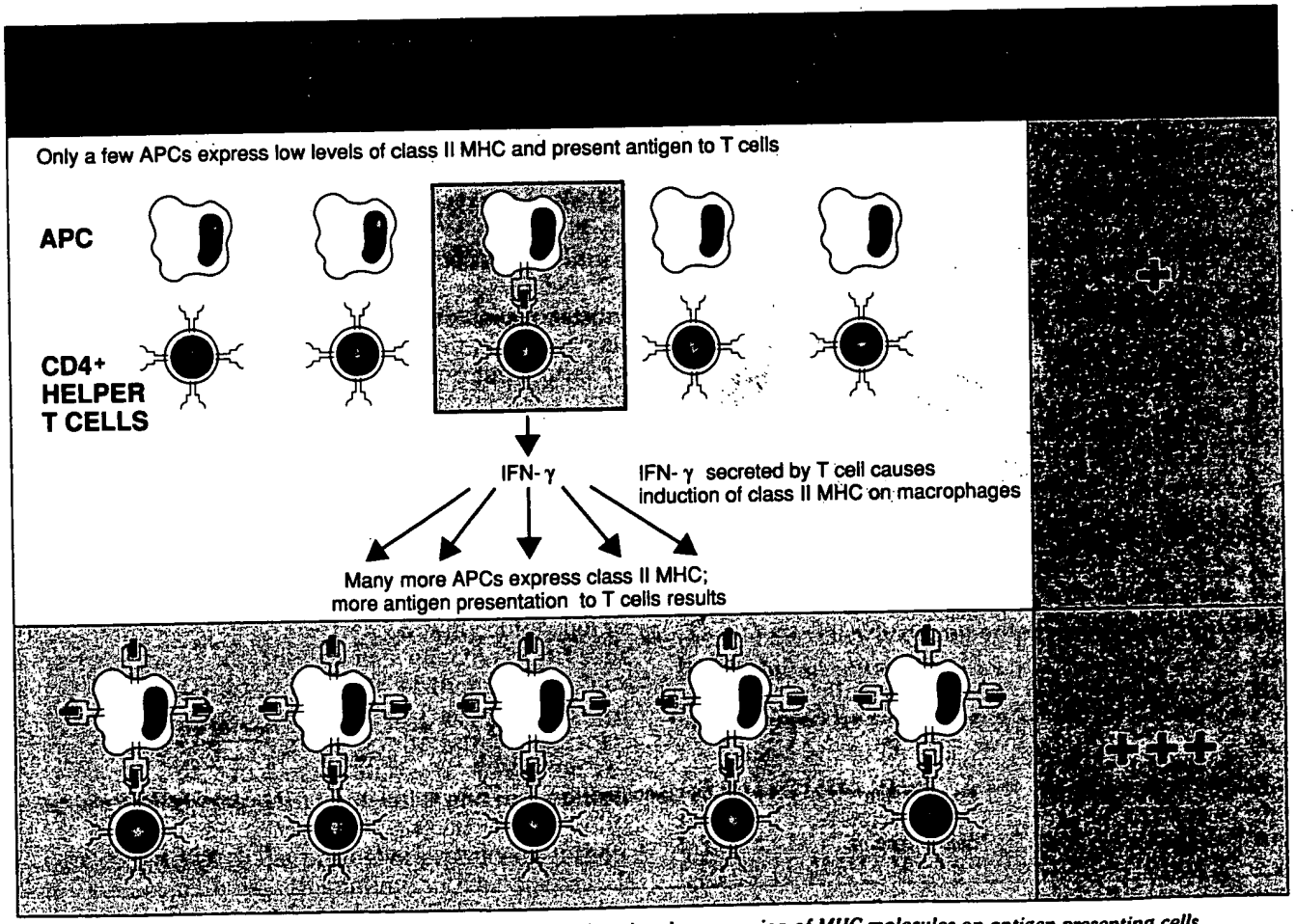


FIGURE 6-4. Gamma interferon amplifies T cell activation by enhancing the expression of MHC molecules on antigen-presenting cells.

tation and T cell activation. Thus, the ability of IFN-γ to stimulate the expression of class II MHC molecules may be an important amplification mechanism for T cell-mediated immune responses.

Since virtually all nucleated cells express class I MHC molecules, all such cells can present endogenously produced foreign protein antigens to class I MHC-restricted CTLs and, therefore, can serve as targets for $CD8^+$ CTLs. This is an important defense mechanism against viruses, which can infect many types of nucleated cells. Various T cell-derived cytokines, particularly IFN-γ and tumor necrosis factor (TNF), augment the expression of class I MHC molecules. Both these cytokines are produced by CTLs, suggesting that cytokine regulation of class I expression may serve to amplify CTL-target interactions.

The mechanisms of antigen processing and presentation have been most thoroughly analyzed for extracellular antigens that are recognized by $CD4^+$ class II MHC-restricted helper T cells. Less is known about antigen presentation to $CD8^+$ class I-restricted CTLs. Therefore, much of the subsequent discussion will focus on the class II-associated presentation of extracellular antigens by APCs. Antigen presentation to CTLs is described separately on p. 131.

Uptake and Processing of Extracellular Protein Antigens by Antigen-Presenting Cells

The initial step in the presentation of a foreign protein antigen is the binding of the native antigen to an APC. Different APCs can bind protein antigens in several ways and with varying efficacies and specificities. Macrophages and dendritic cells bind many different antigens with little or no specificity to surface molecules that are undefined. There are, however, special cases in which the surface molecule on the APC that mediates binding and subsequent internalization of the antigen is identified. For example, specific receptors for the Fc portions of immunoglobulins and receptors for the complement protein C3b, which are present on the surface of macrophages, can efficiently bind opsonized antigens and enhance their internalization. This may partially explain why secondary immune responses require lower doses of antigen than primary responses, since at the time of secondary immunization pre-existing specific antibody may augment binding of the antigen to APCs.

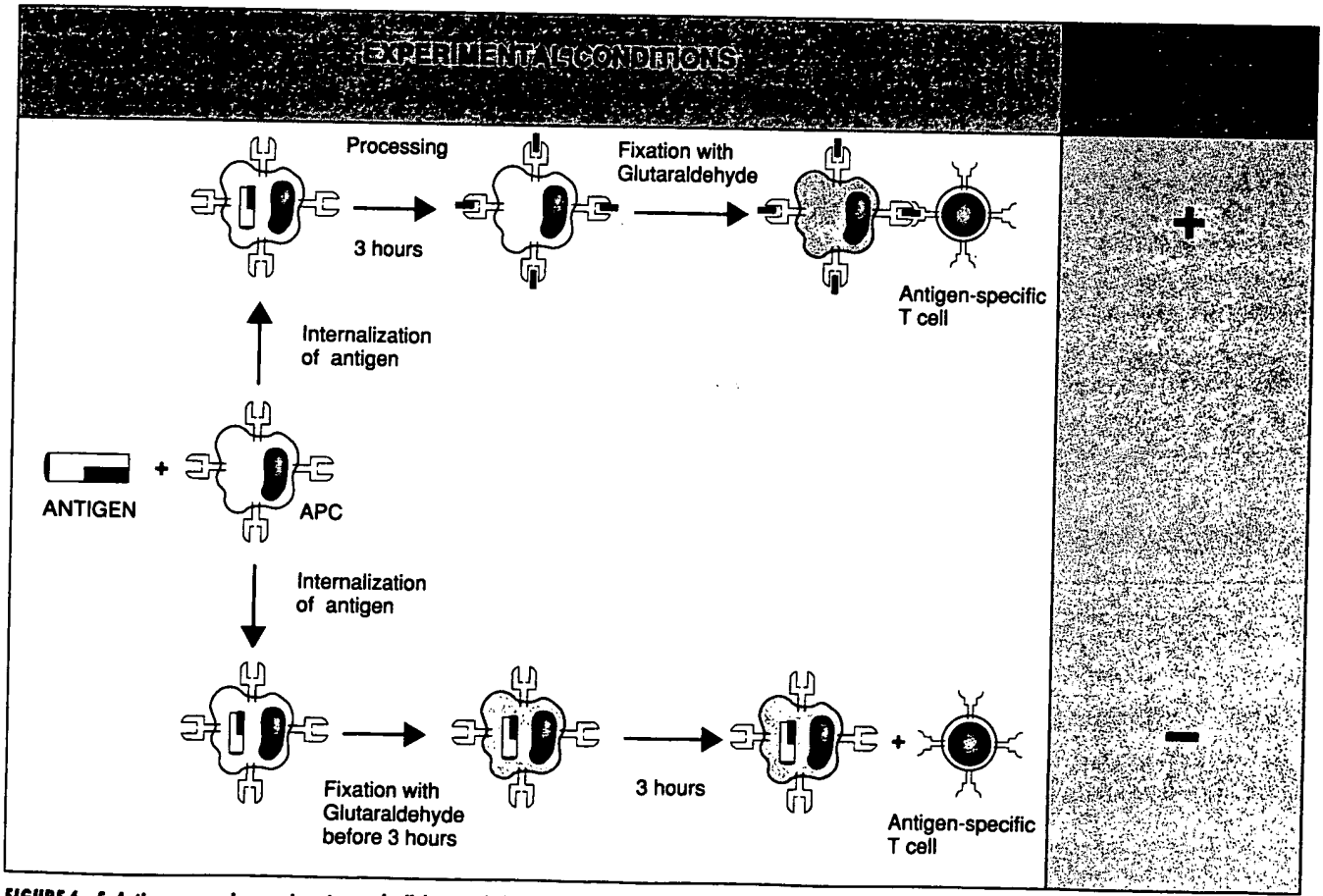


FIGURE 6-5. Antigen processing requires time and cellular metabolism. If an antigen-presenting cell (APC) is allowed to process antigen and is then chemically fixed (rendered metabolically inert) 3 hours or more after antigen internalization, it is capable of presenting antigen to T cells. Antigen is not processed or presented if APCs are fixed less than 1 to 3 hours after antigen uptake. Effective antigen presentation is assayed by measuring a T cell response, such as cytokine secretion.

Another example of specific receptors on APCs is the surface Ig on B cells, which can bind antigens at low concentrations in a highly specific and efficient manner and internalize these antigens for processing.

Within minutes after antigens bind to APCs, they enter the cells, usually by phagocytosis or by receptor-mediated endocytosis in clathrin-coated vesicles. Soluble protein antigens may also be internalized into APCs by fluid phase pinocytosis, without actually binding to the cell surface. Such internalized antigens become localized in intracellular membrane-bound vesicles called **endosomes.** The next step in antigen presentation is the processing of the antigen that was internalized in its native form. Several characteristics of the processing of protein antigens are known:

1. *Antigen processing is a time- and metabolism-dependent phenomenon that takes place subsequent to internalization of antigen by APCs.* If macrophages (or other APCs) are incubated briefly ("pulsed") with a protein antigen such as ovalbumin (OVA), rendered metabolically inert by chemical fixation at various times thereafter, and tested for their ability to stimulate OVA-specific T cells, functional antigen presentation occurs only if 1 to 3 hours elapse between the antigen pulse and fixation (Fig. 6-5). This time is required for the APCs to process the antigen and present it in association with class II MHC molecules on the cell surface. Processing of antigen is inhibited by maintaining the APCs below physiologic temperatures, by adding metabolic inhibitors such as azide, or by fixation before or less than 1 hour after the antigen pulse.

2. *Antigen processing takes place in acidic intracellular compartments.* Chemical agents that increase the pH of intracellular acid vesicles, such as chloroquine and ammonium chloride, are potent inhibitors of antigen processing.

3. *Cellular proteases are required for the processing of many protein antigens.* Protease inhibitors, such as leupeptin, block the presentation of protein antigens by APCs. The function of proteases is to cleave native protein antigens into small peptides. Many cellular proteases function optimally at acid pH, and this is the likely reason why antigen processing occurs best in acidic endosomes.

4. *The processed forms of most protein antigens that T cells recognize are proteolytically cleaved fragments presented on the surface of APCs.* Macrophages that are fixed or that are treated with chloroquine before exposure to antigen can effectively present pre-digested peptide fragments of that antigen, but not the intact protein, to specific T cells (Fig. 6-6). This phenomenon has proved to be extremely useful in analyzing the minimal features of a peptide required for binding to MHC molecules and recognition by T cells. Peptides generated by the *in vitro* proteolysis of a complex globular protein or produced synthetically are capable of stimulating antigen-specific T cells in the presence of fixed APCs, and such peptides can be analyzed for amino acid sequence and secondary structure. Immunogenic peptides derived from many complex globular proteins, such as cytochrome c, ovalbumin, myoglobin, and lysozyme, have been characterized in detail in this way. Immunogenic fragments of **hen egg lysozyme** (HEL), generated by proteolytic cleavage, have been analyzed extensively by Emil Unanue and his associates. We will refer to HEL-derived peptides later in this chapter as prototypical examples for our discussion of the biology of antigen processing and presentation.

In summary, the intracellular proteolysis of endocytosed protein at acid pH generates peptide fragments that can bind to MHC molecules and can be presented to T cells. This type of processing is required for MHC-associated presentation of most protein antigens, because the peptide-binding cleft of MHC molecules is of a size that accommodates peptides that are only 10 to 20 amino acids long (see Chapter 5). Proteolysis may not be necessary for proteins that can bind to MHC molecules in an unfolded configuration even if their size is such that the ends hang out of the binding clefts of MHC molecules. One example of such a protein is fibrinogen, a 30 kD molecule that can be presented by fixed APCs, i.e., without processing. The carboxy terminal end of this protein contains a hydrophilic portion that has no identifiable secondary structure and may be capable of binding to MHC molecules in its native form.

The requirement for antigen processing prior to T cell stimulation explains why T cells recognize linear but not conformational determinants of protein and why T cells cannot distinguish between native and denatured forms of a protein antigen (see Table 6-1). Moreover, in mammalian cells, polysaccharides and lipids cannot be processed to a form that can associate with MHC molecules. This is the reason why polysaccharides and lipids are not recognized by MHC-restricted T lymphocytes and fail to stimulate cell-mediated immunity. It is also likely that most types of APCs, including macrophages, B cells, and dendritic cells, are qualitatively similar in their ability to process endocytosed antigens; however, there may be quantitative differences. For instance, macrophages contain many more proteases than do B cells and are more actively phagocytic, so that macrophages may be more efficient than B cells at internalizing and processing large particulate antigens and presenting peptide fragments of these antigens. It is also possible that different APCs generate distinct sets of peptides from the same native protein because of differences in their endosomal proteases. Although there are no clearly documented examples, this raises the possibility that the APCs involved in presenting a particular protein antigen can influence which T cells are activated by that antigen.

Association of Processed Peptides with Class II MHC Molecules

After protein antigens are processed, they remain sequestered in membrane-bound vesicles and bind to class II MHC molecules within the APCs. The exact site

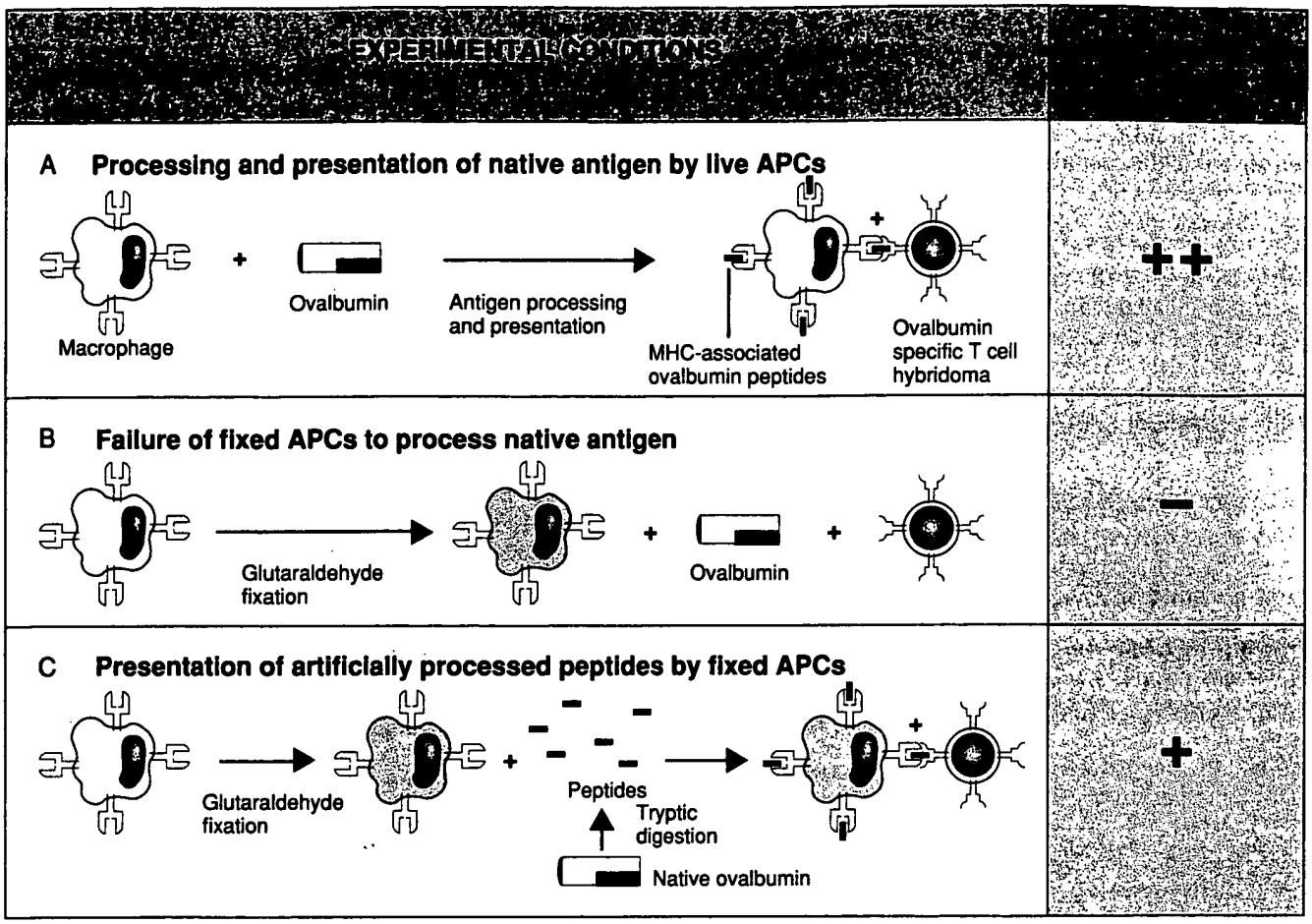


FIGURE 6-6. Metabolically inert (chemically fixed) antigen-presenting cells (APCs) present proteolytic fragments of antigens. *A native antigen is processed and presented by viable APCs* (A) *but not by fixed APCs* (B). *Fixed APCs bind and present proteolytic fragments of antigens to specific T cells* (C). *The artificial proteolysis, therefore, mimics physiologic antigen processing by APCs. (Note that T cell hybridomas respond to processed antigens on fixed APCs, but growth factor-dependent T cells may require costimulators that are destroyed by certain types of fixation. This is discussed fully in Chapter 10.)*

of this association is not known. It has been suggested that class II MHC molecules are synthesized and transported to the cell surface in post-Golgi vesicles. According to this model, the vesicles containing MHC molecules physically intersect endosomes containing processed antigens during their intracellular traffic, and the MHC molecules and processed peptides form non-covalent bonds (Fig. 6-7). The complexes of peptides and class II molecules are then transported to and expressed on the surface of the APCs. It is not known how a protein antigen endocytosed by an APC escapes complete proteolytic degradation during processing and is prevented from entering the lysosomal compartment of the cell. One possibility is that binding of a peptide to an MHC molecule prevents further enzymatic hydrolysis of the peptide. Purified peptides bound to MHC molecules are resistant to destruction by proteases *in vitro*, whereas the same peptides in the absence of MHC molecules are readily hydrolyzed into amino acids by proteolytic enzymes.

The formal experimental demonstration that *antigenic peptides bind to purified MHC molecules even in cell-free solutions* has provided important new approaches for defining the structural basis of MHC-associated antigen recognition. Peptide-MHC binding was first demonstrated by the technique of equilibrium dialysis (see Chapter 3). Purified class II MHC molecules were enclosed within a semipermeable membrane through which they could not diffuse and were incubated with an excess of a freely diffusible fluorescently labeled peptide fragment of HEL. The accumulation of fluorescent label in the compartment containing peptide and MHC molecules was compared with the amount of label in the compartment with peptide alone. Such analyses showed that peptide-MHC binding is saturable, and the dissociation constant (K_d) of the interaction was calculated to be about 10^{-6} M (Fig. 6-8). Other techniques have been developed to measure the physical association of peptides and MHC molecules. These include the use of radioactively labeled peptides and gel filtration to separate unbound peptide from the larger peptide-

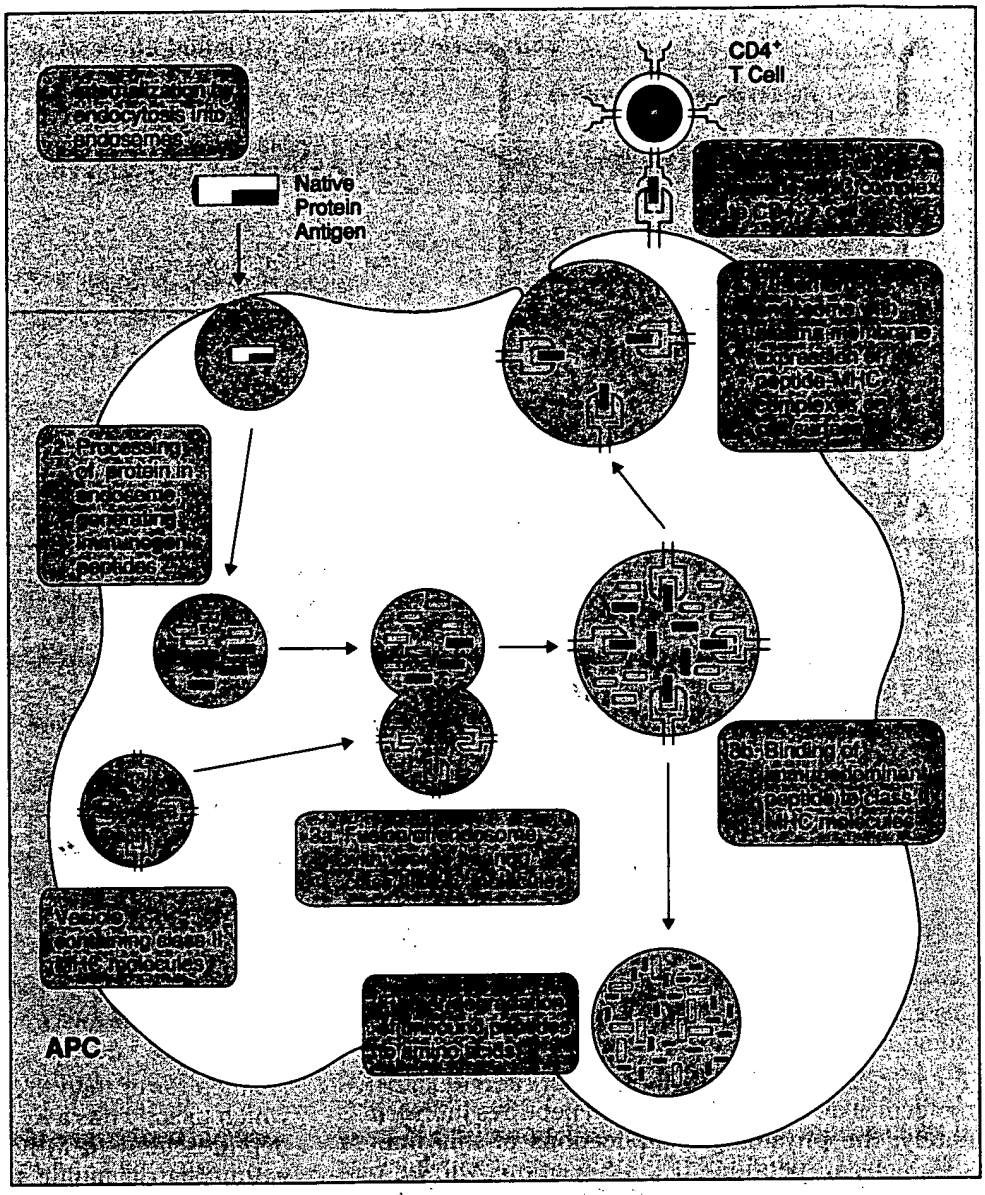


FIGURE 6-7. Pathway of class II MHC-restricted presentation of an extracellular protein antigen.

MHC complexes and the measurement of fluorescence resonance energy transfer between the two sets of interacting proteins labeled with different fluorochromes. Based on such studies, several important characteristics of the binding of antigenic peptides to class II MHC molecules are known. Following are the main features of this association; its physiologic and functional significance are discussed later in the chapter.

1. *Multiple peptide antigens can bind to the same MHC molecule.* This was first suggested by functional assays of antigen competition, before purified MHC molecules were available for direct analyses. For instance, structurally similar amino acid polymers compete with one another for presentation to a T cell specific for either one. Such competition occurs if the competing antigen is added before or together with the antigen that is recognized by the T cell (Table 6-5). These experiments suggested that an MHC molecule can bind more than one antigen but that a T cell is specific for only one of these. Competition between antigens for binding to a particular MHC molecule has been definitively established by direct physical measurements. Thus, many peptides compete with one another for binding to the same MHC molecule. Somewhat surprisingly, these antigenic peptides often show little sequence homology (Table 6-6). Formal proof of a single peptide-binding cleft in each MHC molecule came with the solution of the crystal structure of the HLA-A2 molecule (see Chapter 5). These observations, together with the limited number of MHC alleles expressed in each individual, support the hypothesis that *MHC molecules show a broad specificity for peptide binding, and the fine specificity of antigen recognition resides largely in the antigen receptors of*

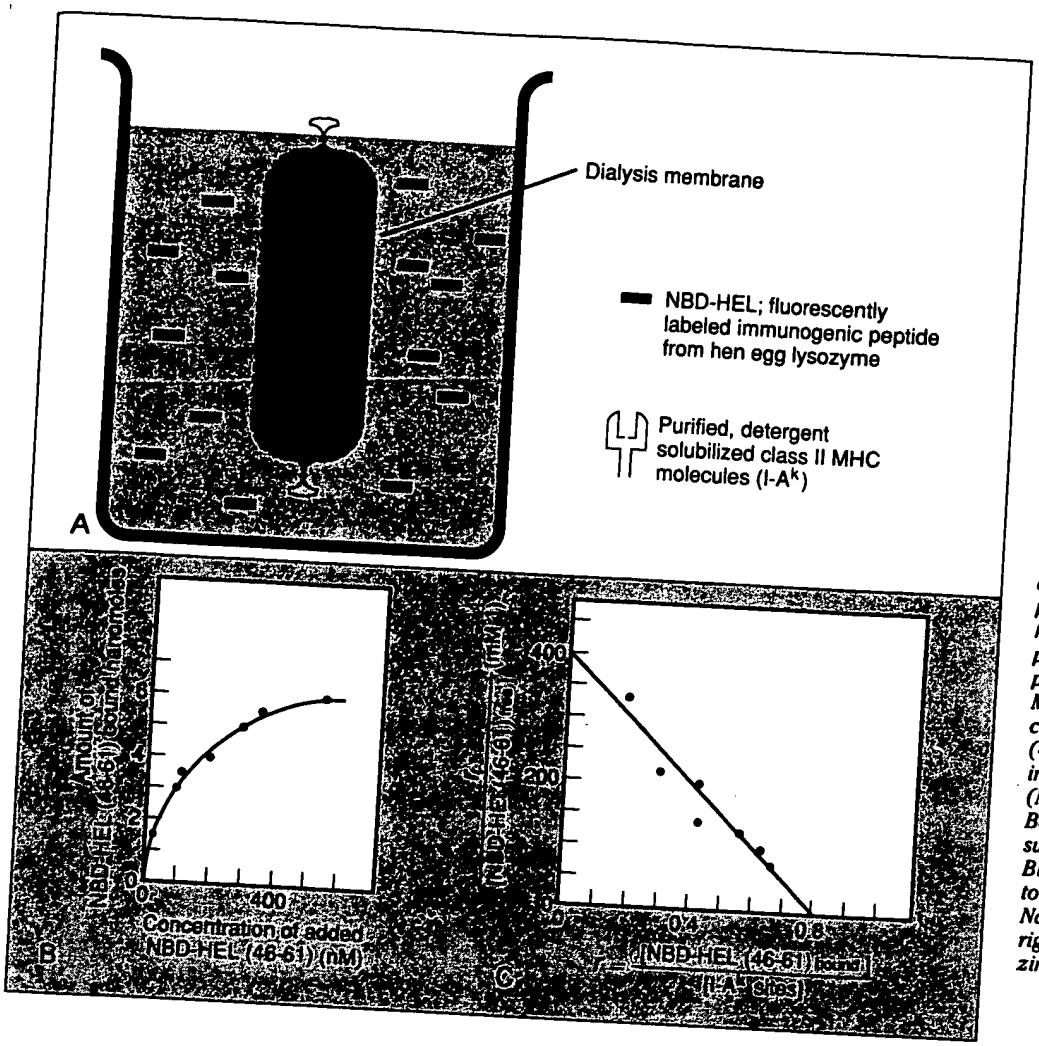


FIGURE 6-8. Demonstration of peptide binding to MHC molecules by equilibrium dialysis. *Purified I-A^k class II MHC molecules bind a peptide fragment of hen egg lysozyme (HEL), HEL (46-61), that is labeled with a fluorescent marker (NBD), which allows the concentration of the peptide to be determined* (A). *Analysis of the amount of HEL bound (i.e., the concentration within the dialysis membrane minus the concentration outside) at varying concentrations of peptide shows that the binding is saturable* (B). *Scatchard analysis shows that each I-A^k molecule has approximately one binding site (X-axis intercept), and the dissociation constant (K_d) is approximately 1×10^{-6} M (calculated from the slope of the line). In this plot, r represents the number of peptide molecules bound to each MHC molecule when there is an excess of peptide* (C). *Note that HEL (46-61) is known to be presented in association with I-A^k molecules. (Modified with permission from Babbitt, B. P., P. M. Allen, G. Matsueda, E. Haber, and E. R. Unanue. Binding of immunogenic peptides to Ia histocompatibility molecules. Nature 317:359-361, 1985. Copyright © 1985, Macmillan Magazines Limited.)*

T lymphocytes. On the other hand, individual MHC molecules do not bind all foreign peptides indiscriminately; the physiologic implications of this limited specificity of peptide-MHC associations will be discussed later.

2. *The association of antigenic peptides and MHC molecules is a low-affinity interaction ($K_d \cong 10^{-6}$ M) with a slow "on rate" and a very slow "off rate."* This affinity is much lower than that of antigen-antibody binding, which usually has a K_d of 10^{-7} to 10^{-11} M. In solution, peptides bind to MHC molecules, reach saturation within 15 to 30 minutes, and remain bound for as long as 6 hours at neutral pH. Dissociation occurs more rapidly at acidic pH. The slow on rate suggests that conformational changes, probably in the peptide, are needed to permit binding. The very slow off rate may

TABLE 6-5. Competition of Antigens for Presentation by Antigen-Presenting Cells (APCs)*

APC	Antigen (Amino Acid Polymer)	Time of Addition of Excess Competing Antigen, GT	Stimulation of GAT-Specific, I-A^d Restricted T Cells
A I-A^d expressing macrophage	GAT	None added	+/No competition
B I-A^d expressing macrophage	GAT	GT same time as GAT	−/Competition
C I-A^d expressing macrophage	GAT	GT 3 hours after GAT	+/No competition

The random amino acid polymer GAT is effectively presented by I-A^d expressing macrophages to GAT-specific, I-A^d restricted T cells, stimulating them to secrete cytokines (A). When an excess of a structurally similar amino acid polymer, GT, is added to the APCs at the same time as the GAT, there is competition so that GAT is not presented and there is no T cell response (B). If, however, GAT is added to the APC first and time is allowed for processing, latter addition of GT does not block presentation of GAT (C). In these experiments, processed forms of both GAT and GT bind to the I-A^d molecule, but only GAT-I-A^d complexes stimulate the T cells.

Abbreviations: GAT, glutamic acid-alanine-tyrosine; GT, glutamic acid-tyrosine.

TABLE 6-6. Binding of Unrelated Peptides to an MHC Molecule: Correlation with Inhibition of Antigen Presentation

Competing Peptide	Ability of Peptide to Compete with OVA(323-339) for Binding to I-A^d	Ability of Peptide to Inhibit Presentation of OVA(323-339) by I-A^d Expressing APC to OVA-Specific, I-A^d Restricted T Cells
Ovalbumin OVA(323-339)	++++	NA
Influenza virus hemagglutinin Ha(130-142)	++++	++++
Hen egg lysozyme HEL(74-86)	++	+++
λ-repressor protein λ-(12-26)	++	++
Sperm whale myoglobin Myo(132-153)	±	±
Herpes simplex virus glycoprotein HSV(8-23)	±	±

Peptide binding to purified MHC molecules correlates with MHC-restricted presentation of peptide to T cells. Binding of ^{125}I-labeled ovalbumin-derived peptide, OVA(323-339), to purified, detergent solubilized murine I-A^d was measured by gel filtration in the presence of varying concentrations of unlabeled peptides. Presentation of OVA(323-339) by paraformaldehyde fixed, I-A^d expressing APC to an OVA(323-339)-specific, I-A^d-restricted T cell line, in the presence of varying concentrations of competing peptides, was assayed by measuring antigen-induced T cell cytokine secretion. The results indicate that the ability of a competing peptide to block OVA(323-339) binding to purified I-A^d correlates well with the ability of the same competing peptide to block I-A^d-restricted presentation of OVA(323-339) to T cells. Numbers following abbreviations of proteins refer to the amino acid residues of the native protein.

Adapted from Buus, S., A. Sette, S. M. Colon, C. Miles, and H. M. Grey: The relationship between major histocompatibility (MHC) restriction and the capacity of Ia to bind immunogenic peptides. Science 235:1353-1358, 1987. Copyright 1987 by the AAAS.
Abbreviations: MHC, major histocompatibility complex; NA, not applicable.

be responsible for peptide-MHC complexes persisting long enough to interact with T cells, despite the low affinity of peptide-MHC association.

3. *The association of peptides with MHC proteins is determined by the primary and secondary structures of both molecules.* The crystal structure of class I MHC molecules and the model of class II molecules based on the known class I structure were described in Chapter 5 (see Figs. 5-4 and 5-6). The single predicted peptide-binding cleft of a class II molecule has a β-pleated sheet floor and α-helical sides made up of the N-terminal domains of the two chains of the heterodimeric class II protein. Polymorphic amino acid residues are concentrated in this peptide-binding region. Processed antigenic peptides are thought to lie within the cleft in molecular contact with the floor and the α-helical sides. Some peptides may assume α-helical configurations. All immunogenic peptides contain some amino acids that form contacts with the MHC molecule and other amino acids that point away from the cleft and are presumably recognized by T cells. Mutational analysis of antigenic peptides is a useful method for defining which residues bind to MHC molecules and compete with other antigens for presentation and which residues are recognized by T cells (Table 6-7). Such studies indicate that in order to stimulate T cells, peptides must be capable of forming non-covalent bonds with MHC molecules. This binding, however, is not sufficient for immunogenicity because each peptide must contain residues that are recognized by specific T cells as well.

4. *The association of antigenic peptides with MHC molecules is stabilized by the interaction of antigen-specific, MHC-restricted T cells with the peptide-MHC complexes.* This has been directly demonstrated by measuring the strength of interactions between antigenic peptides and MHC molecules in the presence and absence of specific T cells. For instance, a peptide fragment of ovalbumin (OVA) binds to the mouse class II molecule, I-A^d, incorporated in synthetic lipid membranes. The proximity of the two can be estimated by attaching fluorescent labels to each and measuring resonance energy transfer. The resonance energy transfer is markedly increased if an OVA-specific, I-A^d-restricted T cell population is added. Based on these findings it has been postulated that *in vivo* protein antigens are continuously endocytosed by APCs, processed, associated with class II MHC molecules, and expressed on the cell surface. If these surface complexes are not recognized by a T cell, they recycle back into endosomes, where the acidic pH dissociates the peptide from the MHC molecules. The peptide may be degraded in lysosomes, and the MHC molecules may become available for binding another processed antigen. If, on the other hand, the peptide-MHC molecule complex is recognized on the surface of the APCs by an antigen-specific, MHC-restricted T cell, the complex is prevented from recycling because of the stabilizing influence of the T cell antigen receptor. This leads to activation of the T cell.

5. *The bimolecular complex of processed antigen and MHC molecules is the specific ligand for T cell anti-*

TABLE 6-7. Identification of MHC-Binding and T Cell Receptor-Binding Residues in Peptide Antigens

	HEL Peptide												
	Amino Acid Residue Position No.										Stimulation of HEL-Specific T Cells	Binding to Purified I-A^k	Competition with Native HEL for T Cell Stimulation
	52	*53*	*54*	*55*	*56*	*57*	*58*	*59*	*60*	*61*			
1	Asp	Tyr	Gly	Ile	Leu	Gln	Ile	Asn	Ser	Arg	+	+	NA
2	Asp	Tyr	Gly	Ile	**Ala**	Gln	Ile	Asn	Ser	Arg	−	+	+
3	Asp	**Ala**	Gly	Ile	Leu	Gln	Ile	Asn	Ser	Arg	−	+	+
4	Asp	Tyr	Gly	**Ala**	Leu	Gln	Ile	Asn	Ser	Arg	−	−	−
5	Asp	Tyr	**Ala**	Ile	Leu	Gln	Ile	Asn	Ser	Arg	+	+	NA

Synthetic peptides were produced that differed from the native hen egg lysozyme peptide HEL(52-61) (peptide 1) by substitutions for single residues, and the functional consequences of these engineered mutations were analyzed. Substitutions at positions 56 and 53 (peptides 2 and 3) result in loss of T cell stimulation, but retain I-A^k binding. The amino acids at these positions in the native peptide are part of the epitope recognized by the T cell receptor. Substitution of residue 55 (peptide 4) results in loss of T cell stimulation and I-A^k binding. This residue is in part of the peptide that binds to the class II MHC molecule. A substitution at position 54 (peptide 5) has no effect, and therefore this residue is not essential for binding of the peptide to either the MHC or T cell receptor molecules.

Adapted from Unanue, E. R., and P. M. Allen: The basis for the immunoregulatory role of macrophages and other accessory cells. Science 236:551-557, 1987. Copyright 1987 by the AAAS.

Abbreviations: MHC, major histocompatibility complex; HEL, hen egg lysozyme; NA, not applicable.

gen receptors. Complexes of OVA peptide + I-A^d incorporated in synthetic lipid membranes induce the activation of OVA-specific, I-A^d-restricted monoclonal T cell hybridomas. Similar results have been obtained with many other antigen-MHC combinations.

6. *Peptide-MHC complexes are produced in intact cells as they are in cell-free experimental systems.* If an APC is incubated with a radioactively labeled protein antigen, radioactive complexes composed of antigen fragments and class II MHC molecules can be isolated from the plasma membranes of the APC.

7. *Autologous peptides bind to self MHC molecules.* Equilibrium dialysis experiments (Fig. 6-8) first showed that the binding of an HEL peptide to the mouse class II molecule, I-A^k, could be competitively inhibited by a homologous mouse lysozyme peptide. Complexes of autologous peptides with self MHC molecules are also formed *in vivo.* This was formally demonstrated using two MHC identical strains of mice that express different allelic versions of the hemoglobin β chain, called *Hbb*^{d} and *Hbb*^{s}. Cloned T cell lines specific for *Hbb*^{d} were generated from *Hbb*^{s} mice after immunizing them with hemoglobin from the *Hbb*^{d} strain. Freshly isolated macrophages from unimmunized mice of the *Hbb*^{d} strain could activate these T cells specific for *Hbb*^{d} without the addition of hemoglobin (Fig. 6-9). Since no exogenous hemoglobin was added, processed class II MHC-associated self hemoglobin must have been present on the surface of the macrophages of the *Hbb*^{d} strain.

These findings, that self peptides can be bound by MHC molecules, raise two important questions. First, if individuals process their own proteins and present them in association with their own MHC molecules, why do we normally not develop immune responses against self proteins? It is likely that self-tolerance is mainly due to the absence of lymphocytes capable of recognizing and responding to self antigens, and this is why self peptide-MHC complexes do not induce autoimmunity (see Chapter 8). Second, if MHC molecules are bathed in processed autologous proteins, how can they ever bind and present foreign antigenic peptides? It has been postulated that the constant recycling of MHC molecules from the cell surface to acidic endosomes and back again ensures that self MHC molecules will be stripped of bound self peptides long enough to allow binding of foreign peptides. Engagement of the T cell receptor may stabilize peptide-MHC interactions, as discussed above, and this can happen only with foreign peptide-self MHC complexes, since T cells normally recognize only foreign antigens.

In summary, the principal steps in class II MHC-associated antigen presentation (see Fig. 6-7) are the following:

1. Internalization of native protein antigens from the extracellular environment into APCs.
2. Processing of the antigen in acidic endosomes, leading to the generation of peptide fragments.
3. Low-affinity binding of peptides to class II MHC molecules within the APCs.
4. Expression of peptide-MHC complexes on the cell surface.
5. Recognition of the complexes by T cells that are specific for the foreign peptide and the self MHC molecule.

The broad specificity of MHC molecules for peptides accounts for the ability of an APC with a limited number of allelic forms of MHC proteins to present many different antigens.

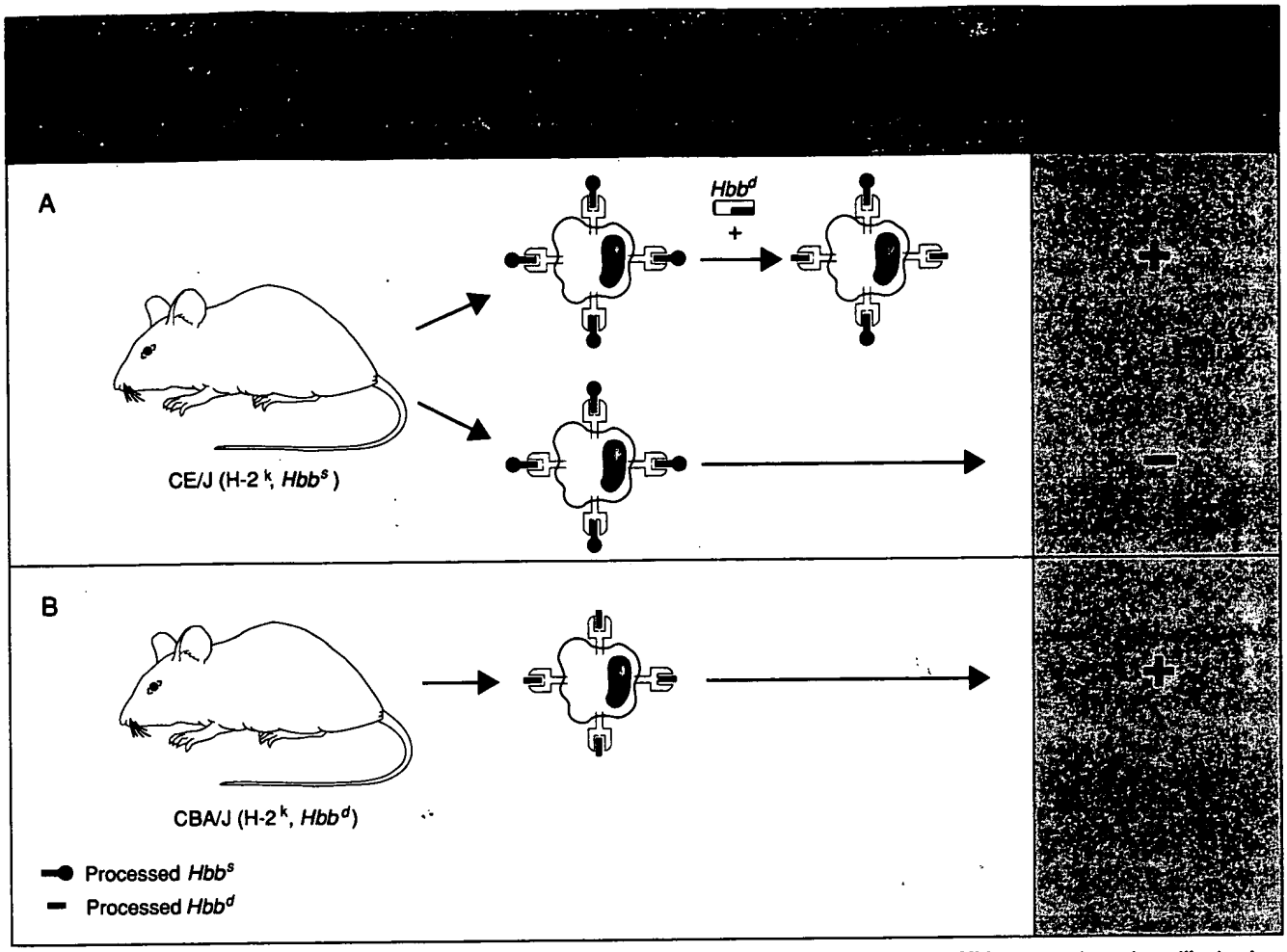


FIGURE 6-9. Macrophages present self hemoglobin in association with self MHC molecules in vivo. *Macrophages from* Hbb^s*-expressing mice will stimulate* Hbb^d*-specific I-A^k-restricted T cells only if the antigen,* Hbb^d*, is added (A). However, freshly isolated macrophages from* Hbb^d*-expressing mice stimulate the same T cells without any requirement for exogenous antigen (B). Therefore, the freshly isolated macrophages from* Hbb^d*-expressing mice must bear processed* Hbb^d *on their surface.*

Class I MHC-Associated Antigen Presentation

As we have mentioned previously, CD8$^+$ T cells, most of which are CTLs, recognize class I MHC-associated foreign protein antigens. Furthermore, CD8$^+$ T cells usually recognize protein antigens that are synthesized within APCs and subsequently expressed on the surface in association with class I MHC molecules. Examples of endogenously synthesized foreign proteins are viral proteins and tumor antigens. CTLs are the principal immunologic defense mechanisms against viruses and may be important in the immune destruction of tumors.

In vitro analyses of the binding of foreign peptides to class I MHC molecules indicate that it is essentially similar to peptide-class II binding. Each class I MHC molecule has a single binding cleft that accommodates peptides that are 10 to 20 amino acids long. The affinity of class I-peptide association is in the order of 10^{-6} M. Different peptides can bind to the same site in a class I MHC molecule and compete with one another for presentation. Any one peptide can also bind to class I and class II MHC molecules, and there are no structural motifs that confer specificity for class I or class II MHC association on an antigenic peptide. Whether a particular antigen will be presented by an APC in association with class I or class II MHC molecules is apparently determined by the intracellular compartmentalization of the protein. Antigens that are synthesized endogenously within the APCs generally traverse different compartments from those antigens that are endocytosed from the extracellular environment. This is supported by several studies:

1. If a viral protein, such as influenza nucleoprotein, or a protein like ovalbumin is added in soluble form to a cell that expresses class I and class II MHC molecules, the antigen is internalized, processed, and presented only in association with class II MHC molecules. Such exogenously added antigens will be recognized by class II-restricted, antigen-specific CD4$^+$ T cells but will not sensitize the APC to lysis by CD8$^+$ cells. On the other hand, if the gene encoding the vira

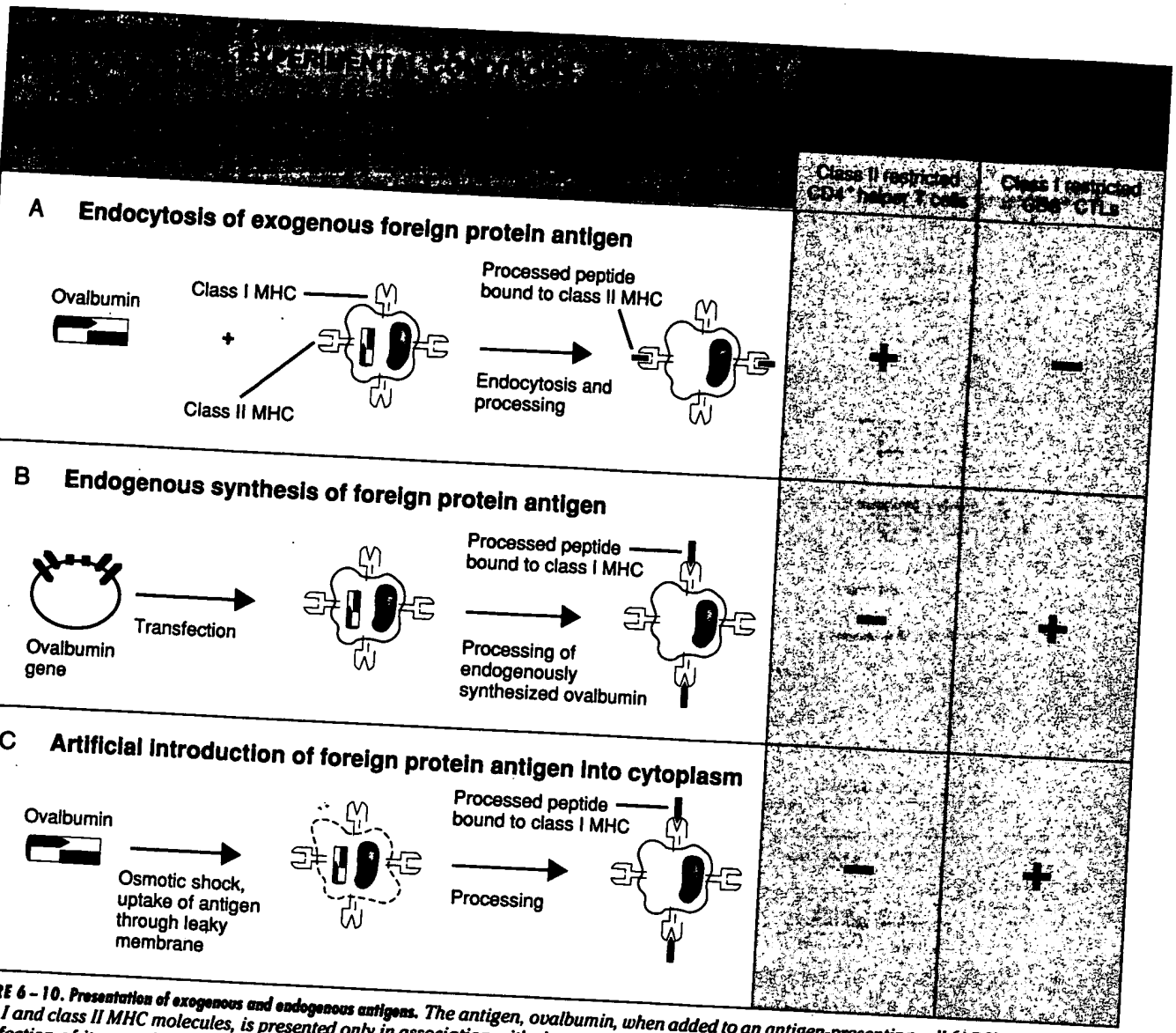


FIGURE 6-10. Presentation of exogenous and endogenous antigens. *The antigen, ovalbumin, when added to an antigen-presenting cell (APC) that expresses class I and class II MHC molecules, is presented only in association with class II (A). The same ovalbumin synthesized intracellularly as a result of transfection of its gene (B) or introduced into the cytoplasm by osmotic shock (C) is presented in association with class I MHC molecules. The measured response of class II restricted helper T cells is cytokine secretion and the measured response of class I-restricted cytolytic T lymphocytes (CTLs) is killing of the APCs.*

protein or ovalbumin is transfected into the APCs so that the antigen is synthesized endogenously, the cell becomes sensitive to lysis by specific class I-restricted $CD8^+$ cells (Fig. 6-10). APCs that express these transfected gene products do not stimulate $CD4^+$ T cells. Intracellular localization rather than endogenous synthesis may be the critical factor determining the class I association of antigenic peptides. For instance, if an antigen is introduced into the cytoplasm of a cell by making the plasma membrane transiently permeable to macromolecules, the antigen is subsequently processed and peptides associate only with class I MHC molecules (Fig. 6-10). This further supports the concept that the traffic patterns of intracellular and endocytosed proteins are different.

2. Presentation of some endogenously synthesized viral antigens to $CD8^+$ CTLs cannot be inhibited by chloroquine, whereas presentation of exogenously added viral proteins to $CD4^+$ T cells is chloroquine-sensitive. This suggests that processing of endogenously produced antigens may not occur in acidic endosomes.

3. *Class I-restricted presentation of endogenously synthesized viral antigens requires association of antigen with newly synthesized class I MHC molecules in the endoplasmic reticulum (ER).* This has been demonstrated in two ways. First, a pharmacologic inhibitor of protein transport out of the ER, called Brefeldin A, can block the post-translational processing and transport of all newly synthesized proteins including self class I and class II MHC and foreign viral proteins. This drug treatment inhibits the class I-restricted presentation of endogenously synthesized viral protein, more than the class II-restricted presentation of

exogenously encountered proteins. Second, the adenovirus E19 protein specifically binds to and prevents transport of class I MHC molecules out of the ER. The ability of E19 to block nascent class I transport correlates with its ability to block class I-restricted antigen presentation.

Thus, the association of antigens with class I versus class II MHC molecules is due to the trafficking of the antigens through different intracellular compartments (Fig. 6-11). In most cases, the commitment to one or another traffic pattern is determined by where the antigen comes from; endogenously synthesized antigens end up associated with class I MHC and exogenously synthesized and endocytosed antigens end up associated with class II MHC. There are exceptions, however, when endogenously synthesized proteins do end up being presented in association with class II MHC molecules.

Many unanswered questions remain about the cell biology of class I-restricted antigen presentation. The site at which antigens are processed into peptides before association with class I MHC molecules is not known, nor is it understood how the processed peptides get into the rough ER, where nascent class I MHC molecules are being synthesized. There is some evidence that the ER itself may contain proteolytic enzymes that could generate immunogenic peptides. Since both class I and class II MHC molecules are produced in the rough ER and both have a natural affinity for peptides, there must also be some mechanism that prevents peptides from binding to class II molecules in the ER. This may be accomplished by the class II-associated invariant or γ chains that may interfere with the peptide-binding clefts. Class I MHC molecules do not have invariant chains when they are synthesized and are therefore free to bind peptides produced within the cell. This may be one reason why

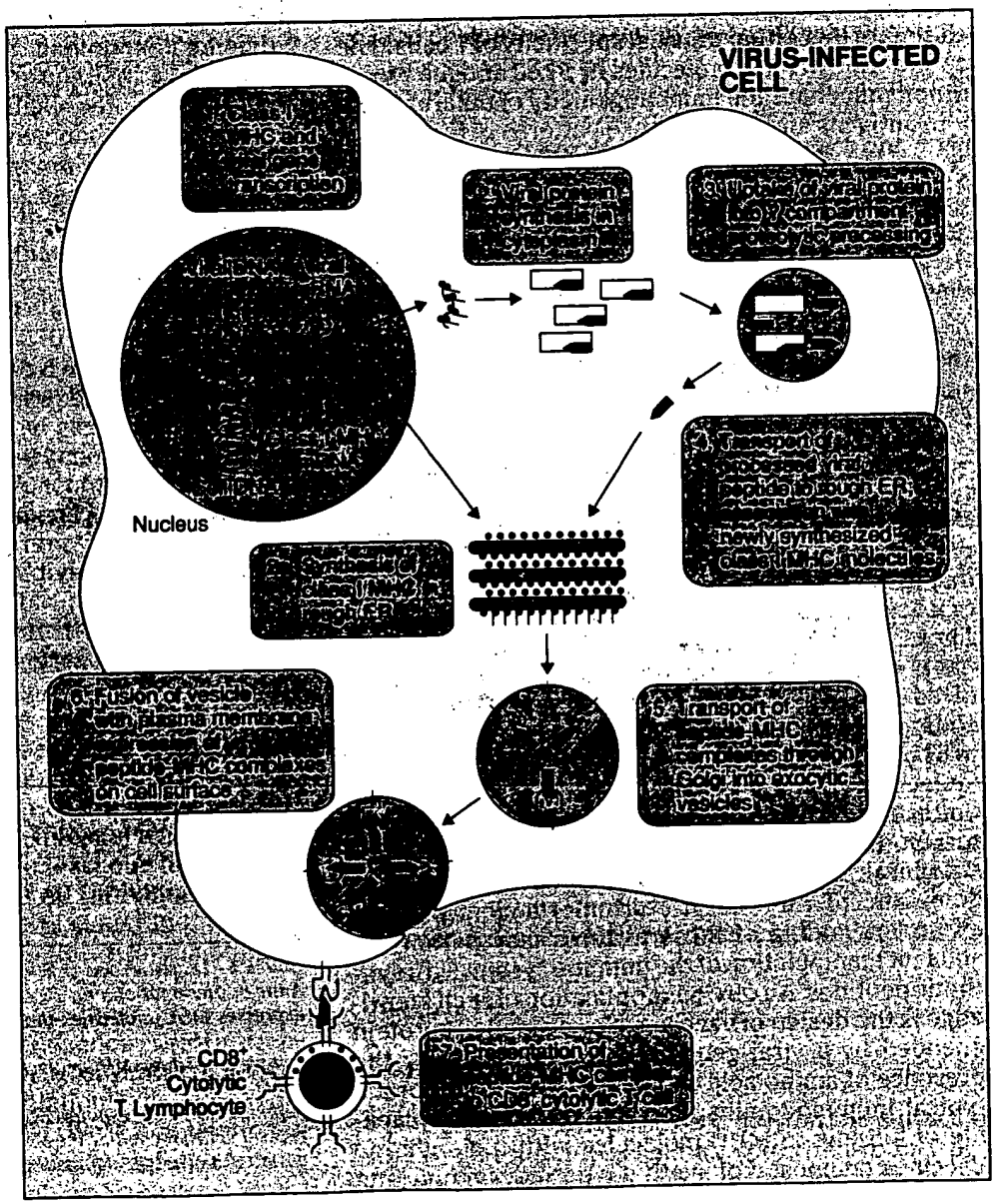


FIGURE 6-11. Pathway of class I MHC-restricted presentation of an endogenously synthesized (e.g., viral) antigen.

endogenously synthesized antigens become class I-associated. As the class II MHC molecules are being transported in vesicles to the cell surface, they shed the invariant chain. At this stage, the vesicles containing MHC molecules may intersect with endosomes containing internalized, processed antigens. Since the binding clefts of the class I molecules are already occupied by peptides, the endocytosed and processed antigens bind to the only available MHC molecules, which are now the class II molecules.

PHYSIOLOGIC SIGNIFICANCE OF MHC-ASSOCIATED ANTIGEN PRESENTATION

So far we have discussed the specificity of CD4+ and CD8+ T lymphocytes for MHC-associated foreign protein antigens and the mechanisms by which complexes of peptides and MHC molecules are produced. It is important to also consider the physiologic implications of this rather unusual specificity of T cells. The central role of MHC molecules in T cell antigen recognition influences the immunogenicity of different protein antigens and the response patterns of the T cells.

Immunogenicity of Protein Antigens

Our present understanding of the role of MHC genes in determining the ability of protein antigens to induce specific immunity is limited largely to exogenous antigens. MHC molecules may determine the immunogenicity of protein antigens in two related ways:

1. *The immunodominant epitopes of complex proteins are often the peptides that bind most avidly to MHC molecules.* If an individual is immunized with a multideterminant protein antigen, in many instances the majority of the responding T cells are specific for one or a few linear amino acid sequences of the antigen. These are called the "immunodominant" determinants or epitopes. For instance, in H-2^k mice immunized with HEL, more than half the HEL-specific T cells are specific for the epitope formed by residues 46 to 61 of HEL in association with the I-A^k but not the I-E^k molecule. This is because HEL(46-61) binds to I-A^k better than do other HEL peptides and does not bind to I-E^k. However, it is not yet known exactly which structural features of a peptide determine immunodominance. The question is an important one because an understanding of these features may permit the efficient manipulation of the immune system with synthetic peptides. An obvious application of such knowledge is the design of vaccines. For example, a protein encoded by a viral gene could be analyzed for the presence of amino acid sequences that would form a typical immunodominant secondary structure capable of binding to MHC molecules with high affinity. Vaccines composed of synthetic peptides mimicking this region of the protein theoretically would be effective in eliciting T cell responses against the viral peptide expressed on an infected cell, thereby establishing protective immunity against the virus.

2. *The expression of particular class II MHC alleles in an individual determines the ability of that individual to respond to particular antigens.* The phenomenon of immune response (Ir) gene-controlled immune responsiveness was mentioned in Chapter 5. We now know that Ir genes that control antibody responses are class II MHC genes. They influence immune responsiveness in part because various allelic class II MHC molecules differ in their ability to bind different antigenic peptides and, therefore, to stimulate specific helper T cells. For instance, H-2^k mice are re-

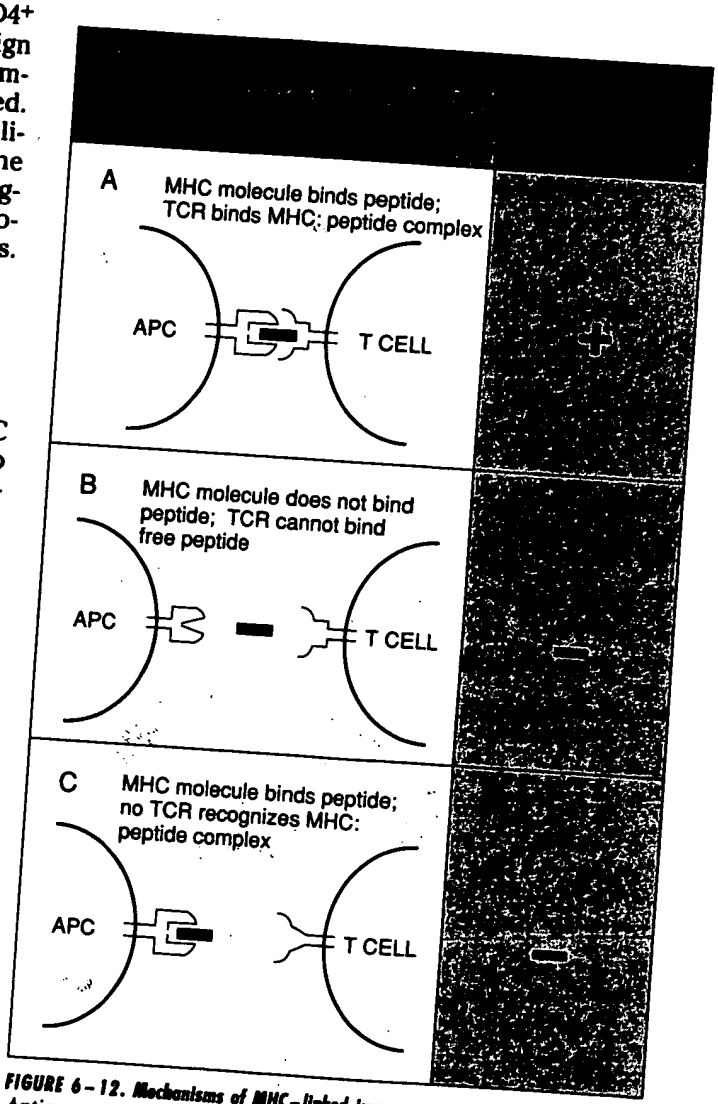


FIGURE 6-12. Mechanisms of MHC-linked immune response (Ir) gene function. *Antigen presentation and T cell activation occur when an individual expresses MHC molecules that can bind peptides derived from the processed antigen and T cells are present that specifically recognize complexes of these MHC molecules with the peptides* (A). *If an individual does not inherit genes encoding MHC molecules that can bind the peptides, no T cell response occurs* (B). *Alternatively, if no T cells are present which recognize the MHC molecules as self, no T cell response occurs* (C). *The development of self-restricted T cells is discussed in Chapter 8.*

sponders to HEL(46-61) but H-2^d mice are non-responders to this epitope. Equilibrium dialysis experiments have shown that HEL(46-61) binds to I-A^k but not to I-A^d molecules (see Fig. 6-8). A possible molecular basis for this difference in MHC association is suggested from the model of the class II molecule and the known amino acid sequences of I-A^k and I-A^d proteins. If the HEL(46-61) peptide in the form of an α-helix is hypothetically placed in the predicted binding cleft of the I-A^k molecule, charged residues of the HEL peptide become aligned with oppositely charged residues of the MHC molecule. This would presumably stabilize the bimolecular interaction. In contrast, the I-A^d molecule has different amino acids in the binding cleft that would result in the aligning of similarly charged residues with the HEL peptide. Therefore, HEL(46-61) would not bind to or be presented in association with I-A^d and the H-2^d mouse would be a non-responder. Similar results have been obtained with numerous other peptides. MHC-linked immune responsiveness may also be important in humans. For instance, Caucasians who are homozygous for an extended HLA haplotype containing HLA-B8,DR3,DQw2a are low responders to hepatitis B virus surface antigen. Individuals who are heterozygous at this locus are high responders, presumably because the other alleles contain one or more HLA genes that confer responsiveness to this antigen. Thus, HLA typing may prove to be valuable for predicting the success of vaccination. These findings support the **determinant selection model** of MHC-linked immune responses. This model, which was proposed many years before the demonstration of peptide-MHC binding, states that the products of MHC genes in each individual select which determinants of protein antigens will be immunogenic in that individual. We now understand the structural basis of determinant selection and Ir gene function in antigen presentation. Most Ir gene phenomena have been studied by measuring helper T cell function, but the same principles apply to CTLs. Individuals with certain MHC alleles may be incapable of generating CTLs against some viruses. In this situation, of course, the Ir genes may map to one of the class I MHC loci.

Although these concepts are based largely on studies with simple peptide antigens and inbred strains of mice, they are also relevant to the understanding of immune responses to complex multideterminant protein antigens in outbred species. It is likely that all individuals will express at least one MHC molecule capable of binding at least one determinant of a complex protein, so that all individuals will be responders to such antigens. As stated in Chapter 5, this may be the evolutionary pressure for maintaining MHC polymorphisms.

This discussion of the influence of MHC gene products on the immunogenicity of protein antigens has focused on antigen presentation and has not considered the role of the T cells. We have mentioned earlier that the exquisite specificity and diversity of antigen recognition are attributable to antigen receptors on T cells. MHC-linked immune responsiveness is also dependent, in part, on the presence and absence of specific T cells. In fact, some peptides bind to MHC molecules in a particular inbred mouse strain but do not activate T cells in that strain (Table 6-7). It is likely that these mice lack T cells capable of recognizing the particular peptide-MHC complexes. *Thus, Ir genes may function by determining antigen presentation or by shaping the repertoire of antigen-responsive T cells* (Fig. 6-12). The development of the T cell repertoire and the role of the MHC in T cell maturation are discussed in Chapter 8.

Nature of T Cell Responses

Based on this knowledge of antigen presentation to T cells, we can now explain other physiologic consequences of MHC-restricted antigen recognition that were introduced in Chapter 5.

1. Because T cells recognize only MHC-associated protein antigens, they can respond only to antigens associated with other cells (the APCs) and are unresponsive to soluble or circulating proteins. *This unique specificity for cell-bound antigens may be essential for the functions of T lymphocytes, which are largely mediated by cell-cell interactions and by cytokines that act at short distances.* For instance, helper T cells help B lymphocytes and activate macrophages. Not surprisingly, B lymphocytes and macrophages are two of the principal cell types that express class II MHC genes, function as APCs for $CD4^+$ helper T cells, and focus helper T cell effects to their immediate vicinity. Similarly, CTLs can lyse any nucleated cell producing a foreign antigen and all nucleated cells express class I MHC molecules, which are the restricting elements for antigen recognition by $CD8^+$ CTLs.

2. The patterns of MHC association of different forms of antigens determine which subset of T cells is preferentially or selectively activated (Fig. 6-13). Extracellular antigens activate class II-restricted $CD4^+$ T cells, which function as helpers to stimulate effector mechanisms such as antibodies and phagocytes that serve to eliminate extracellular antigens. Conversely, endogenous antigens activate class I-restricted $CD8^+$ CTLs, which lyse cells producing these intracellular antigens. *Thus, different forms of antigens selectively stimulate the T cell population that is most effective at eliminating that type of antigen.* This is particularly significant because neither the antigen receptors of helper T cells and CTLs nor class I and class II MHC molecules themselves have the ability to distinguish between extracellular (e.g., bacterial) and intracellular (e.g., viral) protein determinants.

SUMMARY

T cells recognize antigens only on the surface of accessory cells in association with the product of a self MHC gene. $CD4^+$ helper T lymphocytes recognize

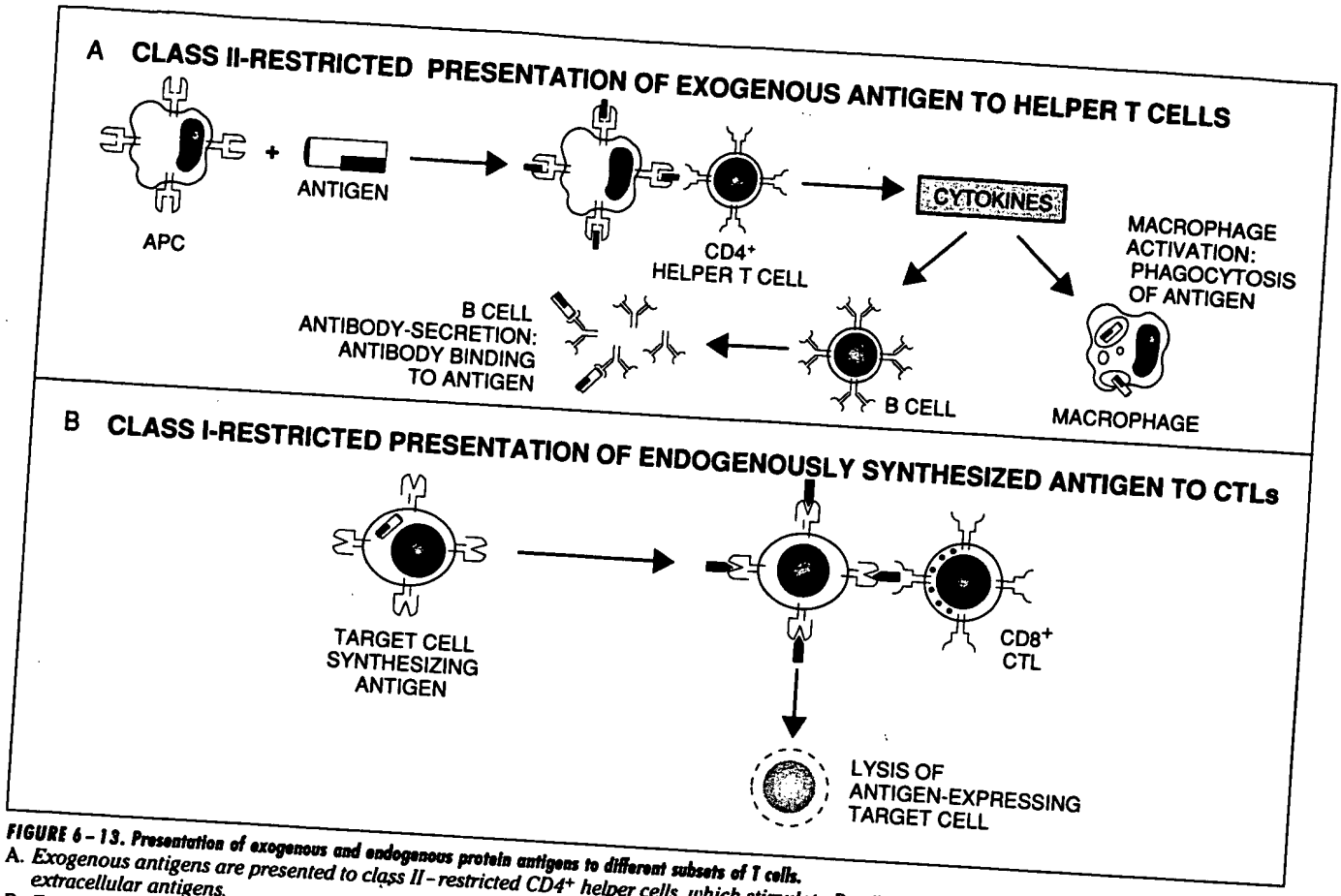


FIGURE 6-13. Presentation of exogenous and endogenous protein antigens to different subsets of T cells.
A. *Exogenous antigens are presented to class II-restricted CD4+ helper cells, which stimulate B cells and macrophages to mediate elimination of extracellular antigens.*
B. *Endogenously synthesized antigens (e.g., derived from intracellular microbes) are presented to class I-restricted CD8+ cytolytic T lymphocytes (CTLs), which kill cells harboring intracellular microbes.*

antigens in association with class II MHC gene products (class II MHC-restricted recognition), and CD8+ CTLs recognize antigens in association with class I gene products (class I MHC-restricted recognition). Exogenous foreign proteins are internalized in APCs, where they undergo processing in an acid vesicular compartment. Processing ensures that portions of a protein (the immunodominant peptides) will bind to class II MHC molecules and form immunogenic complexes. These complexes are expressed on the surface of APCs, where they are recognized by CD4+ T cells. Class II MHC genes function as Ir genes; their products selectively bind and present some antigens but not others. The target antigens for CD8+ CTLs are endogenously synthesized proteins, such as viral antigens, which are processed and associate with class I MHC molecules. Many cell types have the capacity to present antigens to CD4+ helper T cells; a minimal requirement is the expression of class II MHC genes. The regulation of class II MHC gene expression is an important control point for immune responses.

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ORIGINAL COMMUNICATION

T cell vaccination in multiple sclerosis: results of a preliminary study

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Abstract Myelin basic protein (MBP)-reactive T cells are potentially involved in the pathogenesis of multiple sclerosis (MS), and can be depleted by subcutaneous inoculations with irradiated autologous MBP-reactive T cells (T cell vaccination). This preliminary open label study was undertaken to evaluate whether depletion of MBP-reactive T cells would be clinically beneficial to patients with MS. Fifty-four patients with relapsing-remitting (RR) MS (n=28) or secondary progressive (SP) MS (n=26) were immunized with irradiated autologous MBP-reactive T cells and monitored for changes in rate of relapse, expanded disability scale score (EDSS) and MRI lesion activity over a period of 24 months. Depletion of MBP-reactive T cells correlated with a reduction (40%) in rate of relapse in RR-MS patients as compared with the pre-treatment rate in the same cohort. However, the reduction in EDSS was minimal in RR-MS patients while the EDSS was slightly increased in SP-MS patients over a period of 24 months. Serial semi-quantitative MRI examinations suggest stabilization in lesion activity as compared with baseline MRI. The findings suggest some potential clinical benefit of T cell vaccination in MS and encourage further investigations to evaluate the treatment efficacy of T cell vaccination in controlled trials.

Key words multiple sclerosis · T cell vaccination

Abbreviations

EAE – experimental autoimmune encephalomyelitis
PHA – phytohemagglutinin
PBMC – peripheral blood mononuclear cells
MBP – myelin basic protein
MRI – magnetic resonance imaging
MS – multiple sclerosis
RR-MS – relapsing-remitting MS
SP-MS – secondary progressive MS

Introduction

There is growing evidence that autoimmune T cell responses to myelin antigens, including myelin basic protein (MBP), may be engaged in the pathogenesis of multiple sclerosis (MS) [17]. MBP-reactive T cells are found to undergo *in vivo* activation and occur at high precursor frequency in the blood and cerebrospinal fluid of patients with MS [1, 4, 26]. These MBP-reactive T cells produce pro-inflammatory Th1 cytokines (IL-2, TNF-α and γ-interferon) and are thought to facilitate myelin-destructive inflammation in the central nervous system [15, 16]. It has been shown that MBP-reactive T cells can induce experimental autoimmune encephalomyelitis

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(EAE), an animal model for MS [2]. EAE can also be prevented or cured by repeated inoculations with MBP-reactive T cells that have been inactivated by chemical treatment or irradiation, a treatment procedure termed T cell vaccination [3]. It has been demonstrated that T cell vaccination induces regulatory immune responses comprised of anti-idiotypic T cells and anti-ergotypic T cells, which contribute to the treatment effects on EAE and other experimental autoimmune disease models [9, 10].

T cell vaccination has advanced recently to clinical trials in patients with MS based on the hypothesis that depletion of MBP-reactive T cells may improve the clinical course of the disease. In a pilot clinical trial, we demonstrated that vaccination with irradiated autologous MBP-reactive T cell clones elicited CD8+ cytolytic T cell responses that specifically recognized and lyzed MBP-reactive T cells used for vaccination [11, 25]. Three subcutaneous inoculations with irradiated MBP-reactive T cell clones resulted in depletion of circulating MBP-reactive T cells in patients with MS. Depletion of MBP-reactive T cells by T cell vaccination appeared to correlate with clinical improvement, as evidenced by a reduction in rate of relapse, expanded disability scale score (EDSS) and MRI lesion activities in relapsing-remitting patients [11]. Although no conclusion could be made from the pilot trial owing to the limited number of patients studied, the excellent safety profile and the potential clinical benefit encouraged further clinical investigations. This preliminary study was undertaken to investigate whether depletion of circulating MBP-reactive T cells would be clinically beneficial to patients with MS. Twenty-eight patients with relapsing-remitting MS (RR-MS) and 26 patients with secondary progressive MS (SP-MS) were included in this open-label study. Patients received three subcutaneous injections of irradiated autologous MBP-reactive T cells and were monitored for changes in rate of relapse, EDSS and MRI lesion activities over a period of 24 months. The results were compared with pre-study values in a self-paired fashion.

Materials and methods

Patients and the study design

Fifty-four patients with MS were enrolled in this study. The inclusion criteria were clinically definite MS for at least two years, baseline EDSS of 1.5 to 6.5 for RR-MS and 4.0 to 8.0 for patients with SP-MS, and at least one exacerbation in the past two years prior to study entry for the RR-MS cohort. Approximately 25% of the patients failed previously to respond to or tolerate treatment with beta-interferon or Glatiramer, and the remaining patients had not been treated with these agents at least three month prior to entry and throughout the study. The patients had not taken any immunosuppressive drugs, including steroids, at least three months prior to enrolling in the study. Steriods were permitted during the study if an exacerbation occurred. Symptomatic treatments for fatigue, spasticity and bladder complaints were not prohibited. Informed consent was obtained from the patients after explaining the experimental procedures. The protocol was approved by the Institutional Human Subject Committee at Baylor College of Medicine.

All patients were given three subcutaneous injections with irradiated autologous MBP-reactive T cells, and observed for time to onset of confirmed progression of disability, EDSS, rate of relapse and MRI lesion activities. The results were compared with the patient's own pre-treatment course. Time to progression was determined by an increase of at least 1.0 on the EDSS [13] persisting for at least 3 months. On-study exacerbations were defined by the appearance of new neurological symptoms or worsening of pre-existing neurological symptoms lasting for at least 48 hours, accompanied by objective change on neurological examination (worsening of at least 0.5 point on EDSS). Patients were instructed to report events between the scheduled regular visits, and were examined by a neurologist if symptoms suggested an exacerbation. Safety assessments included adverse events, vital signs and physical examinations at regular visits.

Estimation of the frequency of MBP-reactive T cells in the blood

The method was described previously [11, 25, 26]. In each case, the material used for cell processing and cell culture was strictly autologous. Peripheral blood mononuclear cells (PBMC) were prepared from heparinized venous blood by Ficoll gradient separation. PBMC were plated out at 200,000 cells/well in the presence of MBP (40 μg/ml) and two synthetic MBP peptides corresponding to the immunodominant regions (residues 83–99 and residues 151–170), respectively, at a concentration of 20 μg/mL. The cell number per well had been predetermined as an optimal cell density to detect MBP-reactive T cells. Seven days later, all cultures were restimulated with autologous PBMC pulsed with MBP and the MBP peptides, respectively. Pulsing of PBMC was carried out by incubating PBMC with the peptides or MBP (100 μg /ml) at 37°C for three hours. Pulsed PBMC were then irradiated at 4,000 rads before use. After another week, each culture was examined for specific proliferation to MBP and the MBP peptides in proliferation assays. Briefly, each well was split into four aliquots (approximately 10^4 cells per aliquot) and cultured in duplicate with 10^5 autologous PBMC in the presence and the absence of MBP and the MBP peptides. Cultures were kept for three days and pulsed with [^{3}H]-thymidine (Amersham, Arlington Hights, IL) at 1 μCi per well during the last 16 hours of culture. Cells were then harvested using an automated cell harvestor and [^{3}H]-thymidine incorporation was measured in a betaplate counter. A well/culture was defined as specific for the peptides when the CPM were greater than 1,500 and exceeded the reference CPM (in the absence of the antigens) by at least three times. The frequency of MBP-reactive T cells was then estimated by dividing the number of specific wells by the total number of PBMC seeded in the initial culture for each antigen [11, 25, 26]. The second precursor frequency analysis of MBP-reactive T cells was performed using the whole MBP as the antigen 2–3 months after the completion of T cell vaccination. The same method of calculation was used consistently to compare the changes of the T cell frequency throughout the study.

Generation of MBP-reactive T cell clones

The resulting T cell lines either specific for the MBP peptides or MBP were selected for cloning. These T cell lines were CD4+/CD8 – and exhibited Th1-like phenotype producing TNF-α and γ-interferon but little or no IL-4 and IL-10. They were cloned subsequently by the limiting dilution assay [11, 25, 26]. Briefly, cells of each T cell line were plated out under limiting dilution conditions at 0.3 cell per well and cultured with 10^5 irradiated autologous PBMC and 2 μg/ml of PHA (Sigma, St. Louis, MO). Cultures were fed every three to four days with fresh medium containing 50 IU /ml of recombinant IL-2 (Chiron, San Diego, CA). After approximately 10–12 days, growth-positive wells became visible and were tested in proliferation assays to select T cell

clones specific for the peptides of MBP. The medium used for T cell culture and cell processing was RPMI 1640 (Hyclone, Logan, UT) supplemented with 10% heat-inactivated autologous serum and 50 IU/ml of recombinant IL-2.

T cell vaccination protocol

The protocol was similar to that used in previous clinical studies [11, 25]. Briefly, MBP-reactive T cell clones were pre-activated with PHA (1 μg/ml) in the presence of irradiated PBMC as a source of accessory cells. Cells were cultured for 5–6 days in RPMI1640 media supplemented with 10% heat-inactivated autologous serum and 50 units of rIL-2. Activated MBP-reactive T cells were subsequently washed three times with sterile saline to remove residual PHA and cell debris. After irradiation (8,000 rads, ^{60}Co source), cells were resuspended in 2 ml of saline and injected subcutaneously on two arms. The number of T cells used for vaccination ranged from 30×10^6 to 60×10^6 cells per injection (2–4 T cell clones used for each injection) and was chosen by an extrapolation of T cell doses effective in experimental animals on the basis of relative skin surface areas [3]. Each patient received three subcutaneous injections at two-month intervals.

Magnetic resonance imaging studies

Magnetic resonance imaging (MRI) was performed as gadolinium-enhanced T1 images. Areas of higher signal intensity were scored in a semiquantitative fashion [14, 19]. This scoring method produced a score related to both the size and number of foci with increased signal hyperintensity. Signal hyperintensities were scored in the following regions: (i) periventricular, in the frontal and occipital region and parallel to the lateral ventricles; (ii) lobar white matter, separately in the frontal, temporal, parietal and occipital region; (iii) the basal ganglia, caudate nucleus,putamen, globus pallidus and thalamus and (iv) the infratentorial region, cerebellum, mesencephalon, pons and medulla. The lesions were graded as follows: a lesion with a diameter less than 0.5 cm was given the score of '1', between 0.5 cm and 1.0 cm as '2', between 1.0 cm and 1.5 cm as '3', between 1.5 cm and 2.0 cm as '4' and greater than 2.0 cm as '5'. The confluent lesions were measured as follows: a score of '5' is given when less than 25% of the region of interest as defined above was considered to be of abnormal signal intensity, '10' and '15' for 25% and 50% when more than 50% of the visualized region of interest was affected. These values were then added to the 'individual' lesion scores.

Statistical analysis

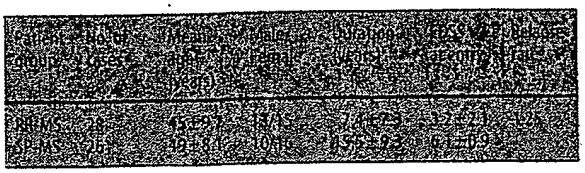
The significance in the precursor frequency of MBP-reactive T cells before and 2–3 months after vaccination was analysed by Student's *t* test. Time to onset of confirmed progression was analysed using Kaplan-Meier method. The differences in the clinical variables in study patients before and after T cell vaccination were analysed with the Wilcoxon's rank-sum test.

Results

The depletion of MBP-reactive T cells by T cell vaccination

Fifty-four patients with RR-MS (n=28) and SP-MS (n=26) were recruited for this open-label study. The baseline clinical characteristics of the patients are shown in Table 1. Each patient received three courses of

Table 1 Pre-treatment clinical characteristics of the patients



subcutaneous injections with irradiated autologous MBP-reactive T cell clones at two-month intervals. Patients were monitored for changes in the precursor frequency of MBP-reactive T cells, rate of relapse, EDSS and MRI lesion activities over a period of 24 months. The results were compared with pre-vaccination values in a self-paired manner.

As is shown in Fig. 1, the precursor frequency of circulating MBP-reactive T cells detected in these MS patients at baseline (14×10^{-5}) was highly comparable with that reported in previous studies (approximately 10×10^{-5} in peripheral blood mononuclear cells) [11, 25]. No significant difference was found in the precursor frequency of MBP-reactive T cells between RR-MS and SP-MS cohorts. More than 90% of the patients developed significant T cell responses to the immunizing T cells after the second and the third vaccination (data not shown). The T cell frequency was undetectable in 92% of patients or declined substantially in the remaining patients 2–3 months after the completion of three courses of vaccination (14×10^{-5} vs. 1.9×10^{-5}, $p < 0.001$). The results confirmed depletion of MBP-reactive T cells by T cell vaccination in patients with MS.

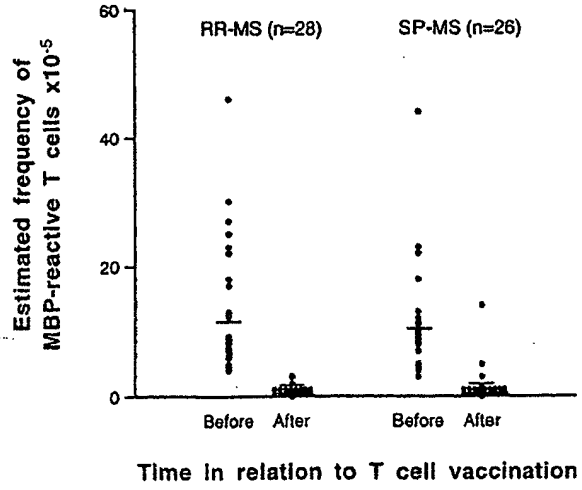
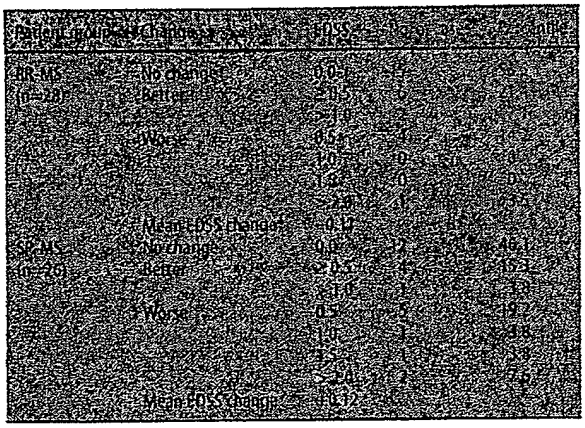


Fig. 1 The changes in the estimated precursor frequency of circulating MBP-reactive T cells before and after vaccination.
The precursor frequency of T cells specific for MBP was estimated in all patients before and 2–3 months after the completion of T cell vaccination.

Changes in EDSS and time to confirmed progression after depletion of MBP-reactive T cells

We attempted to address whether depletion of circulating MBP-reactive T cells by T cell vaccination would alter the clinical course of MS. Except for mild and transient erythema at the injection site seen in some patients, no adverse effects were associated with T cell vaccination, and all patients were treated in an outpatient clinic. As shown in Table 2, the mean EDSS declined slightly in patients with RR-MS (3.21 at entry vs. 3.1 at exit) over a period of 24 months after vaccination. Only one patient (3.5 %) in the treated RR-MS group had progressed beyond EDSS of 2.0 within 24 months. In the SP-MS cohort, mean EDSS progressed slightly (+0.12) over a period of 24 months. Furthermore, as illustrated in Fig. 2, estimation of time to confirmed progression using the Kaplan-Meier method showed 20 % progression in 18 months for both treated groups. However, progression seemed to accelerate after 18 months (12 months after the last vaccination) in both study groups.

Table 2 Amount of sustained change in EDSS to 2 years



[a] Within-person change in EDSS from baseline to year 2.

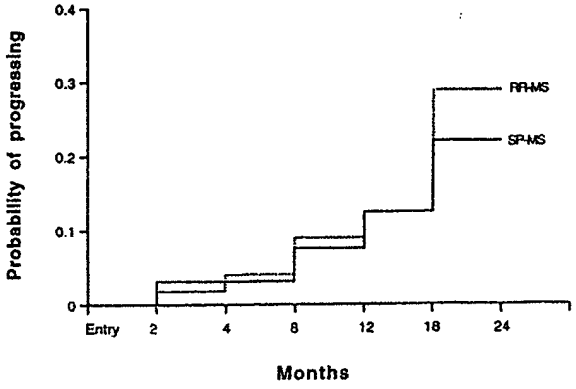


Fig. 2 Kaplan-Meier failure-time curve showing the cumulative probability of progression in relation to number of months to the onset of confirmed disability.

Changes in rate of clinical exacerbation

As shown in Table 3, annual rate of relapse declined in patients with RR-MS after T cell vaccination, representing a 40 % reduction from the baseline relapse rate. The proportion of patients exhibiting no attack was 39 %. No significant difference in the rate of relapse could be found between the first year and the second year of the study. Although the rate of relapse decreased by 50 % in the SP-MS cohort, it was difficult to evaluate the significance of the change as only a small number of the secondary progressive patients examined here (6/26) had a relapse during the two years prior to study entry.

Brain lesion activities by magnetic resonance imaging examinations

Three MRI examinations (gadolinium-enhanced T1 images) were performed at entry (baseline), 12 months and at exit (24 months) to monitor changes in the brain lesion activities as an index of disease progression. Because of technical incompatibility of some scans performed at different medical centers, MRI scans from only 34 patients could be analysed. All MRI scans were evaluated by an outside neuroradiologist who was not involved in the study. A semi-quantitative scoring method used previously in our pilot study and other related studies was employed to evaluate lesion activity [11, 14, 19]. This scoring method produced a score related to both the size and number of foci with increased signal hyperintensity on T1 images. As shown in Table 4, the results revealed that in 70 % of the patients examined the MRI lesion scores were either unchanged or improved as defined by a reduction of at least one point in the lesion score while the remaining 30 % patients had increased lesion scores during the course of the study. As a group, the changes in the mean MRI lesion score represented a 1.2 % reduction in the first year and an increase of 3.3 % from the baseline MRI in the second year.

Table 3 Frequency of clinical exacerbation

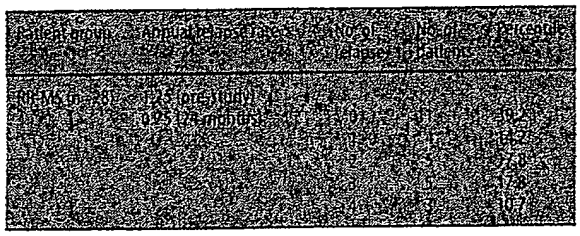
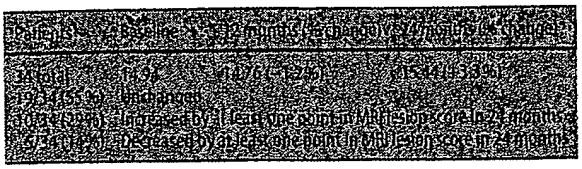


Table 4 Mean MRI lesion score by semi-quantitative analysis and the percent change from baseline MRI.



The changes, however, were not significant ($p > 0.4$). The results may suggest stabilization attributable to T cell vaccination since MRI lesions generally progress by approximately 10% on a yearly basis in non-treated RR-MS patients as documented in previous studies [8, 18]. Taken together, the findings suggest a favorable correlation between the depletion of MBP-reactive T cells by T cell vaccination and some clinical improvement in MS patients examined.

Discussion

Although MBP-reactive T cells undergo *in vivo* activation and clonal expansion and express restricted T cell receptor V gene usage in a given individual, the T cell receptors of MBP-reactive T cells are diverse and vary between different MS patients [6, 20, 21]. Therefore, the current strategy to effectively deplete MBP-reactive T cells in MS patients requires treatment to be individualized. In agreement with the previous studies [11, 25], the present study confirms that vaccination with self MBP-reactive T cells provides a consistent and powerful means of immunizing patients in order to deplete the circulating MBP-reactive T cells. Although the mechanism underlying immune regulation induced by T cell vaccination is not completely understood, it is increasingly clear that T cell vaccination may act on multiple regulatory networks to induce CD8+ anti-idiotypic T cell responses and antibody reactions [7, 25, 27] and Th2 immune deviation [22]. In particular, these anti-idiotypic T cells induced by T cell vaccination were shown to lyse the immunizing T cells in recognition of variable regions of the T cell receptors, which represent the dominant immune regulation responsible for the depletion of MBP-reactive T cells [24]. It is conceivable that these regulatory responses induced by T cell vaccination potentially contribute to the beneficial effect of T cell vaccination in MS.

Although there is indirect evidence suggesting potential association of myelin-reactive T cells with the disease processes in MS [1, 4, 26], it has been extremely difficult, if not impossible, to establish or reject the role of myelin-reactive T cells in the pathogenesis of MS. In this regard, T cell vaccination provides a unique opportunity to assess whether depletion of myelin-reactive T cells has a beneficial impact on the clinical course of MS. The preliminary clinical trial described here suggests a favorable correlation of T cell vaccination with some improved clinical variables. First, the results indicate that depletion of MBP-reactive T cells coincided initially with slow progression in both relapsing-remitting and SP-MS cohorts. However, the disease progression seemed to accelerate 12 months after the last injection. The significance of this apparent accelerated progression is unknown, but it may be associated with a gradual decline of the immunity induced initially by T cell vaccination against MBP-reactive T cells. Indeed, in approximately 10–12% of the immunized patients, MBP-reactive T cells reappeared around that time, supporting this possibility. In some cases, the reappearing MBP-reactive T cells originated from different clonal populations that were not detected before vaccination, which was also observed in the previous studies [27]. The findings suggest that MBP-reactive T cells may undergo clonal shift or epitope spreading [20] potentially associated with the on-going disease processes. If this observation is confirmed, it may indicate the need for additional booster injections with the same or newly appearing T cell clones to maintain adequate immunity, providing important information for improving the current protocol of T cell vaccination. This possibility was explored in a recent study [5].

Annual MRI examinations revealed a slight reduction in MRI lesion activities in the first year and only a 3.3% increase in the second year. The MRI findings may represent stabilization in lesion activity in patients treated with T cell vaccination. There were favorable changes in other clinical variables, including annual rate of relapse and EDSS in vaccinated patients, suggesting a potential beneficial effect of T cell vaccination on the clinical course of MS. The results of the study are largely consistent with the findings reported in the pilot study [11]. However, in contrast to other clinical variables, the impact of T cell vaccination on clinical disability as measured by EDSS was minimal in both study groups. It may reflect the lack of sensitivity of the EDSS to measure changes over a relatively short period of time (24 months). The possibility also exists that even after the autoimmune component is removed or suppressed by T cell vaccination, the inflammatory lesions may still take a long time to resolve and some of the existing tissue damage will be permanent. Moreover, in some patients with advanced disease, the inflammatory lesions may not be directly associated with myelin-reactive T cell responses. Consequently, depletion of MBP-reactive T cells may have little impact on the disease processes in these patients. It is hoped that further investigations may provide new insights into our understanding of these fundamental issues.

The findings described here regarding the treatment efficacy of T cell vaccination should be interpreted with

caution because of the inherent limitations of the study. In the absence of placebo controls, the clinical results were compared with the patient's own pre-treatment status. Such comparisons may introduce biases in the interpretation of the results. The study is also limited by the potential placebo effect associated with the open-label design of the study. Therefore, although the study provided important clinical indications in favor of the potential role of T cell vaccination in MS, the treatment efficacy of T cell vaccination must be evaluated in double-blind and placebo-controlled clinical trials.

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Immunology

Costimulatory function and expression of CD40 ligand, CD80, and CD86 in vascularized murine cardiac allograft rejection

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ABSTRACT **Recent data implicates a role for the CD40–CD40 ligand (CD40L) pathway in graft rejection. One potential mechanism is direct costimulation of T cells through CD40L. Alternatively, the ability of CD40 stimulation to induce CD80 (B7-1) and CD86 (B7-2) expression on antigen-presenting cells (APCs) has led to the hypothesis that the role of CD40–CD40L interactions in transplant rejection might be indirect, i.e., to promote the costimulatory capacity of APCs. Here, we have used a murine vascularized cardiac allograft model to test this hypothesis. Treatment of the recipients with donor splenocytes and a single dose of anti-CD40L mAb induces long-term graft survival (>100 days) in all animals. This is associated with marked inhibition of intragraft Th1 cytokine [interferon γ and interleukin (IL) 2] and IL-12 expression with reciprocal up-regulation of Th2 cytokines (IL-4 and IL-10). In untreated allograft recipients, CD86 is strongly expressed on endothelial cells and infiltrating mononuclear cells of the graft within 24 hr. In contrast, CD80 expression is not seen until 72 hr after engraftment. Anti-CD40L mAb has no detectable effect on CD86 up-regulation, but almost completely abolishes induction of CD80. However, animals treated with anti-CD80 mAb or with a mutated form of CTLA4Ig (which does not bind to CD86) rejected their cardiac allografts, indicating that blockade of CD80 alone does not mediate the graft-prolonging effects of anti-CD40L mAb. These data support the notion that the role of CD40–CD40L in transplant rejection is not solely to promote CD80 or CD86 expression, but rather that this pathway can directly and independently costimulate T cells. These data also suggest that long-term graft survival can be achieved without blockade of either T cell receptor-mediated signals or CD28–CD86 engagement.**

It is well-accepted that T cells require costimulatory signals for optimal activation (1, 2). At present, CD28 is the best-characterized costimulatory receptor on T cells (3, 4). Its known ligands, CD80 (B7-1) and CD86 (B7-2), are expressed on activated antigen-presenting cells (APCs) (3, 4). Blockade of CD28–B7 interactions has been shown to inhibit a number of immune responses *in vitro* and *in vivo*, including transplant rejection, induction of graft versus host disease, and autoimmune syndromes (for review, see ref. 5).

Recently, other costimulatory pathways have been characterized (1). One which has been the subject of intense study is that of CD40–CD40 ligand (CD40L) (6, 7). CD40, a member of the tumor necrosis factor (TNF)-receptor family is expressed on the surface of B cells. T cell activation induces expression of a molecule of the TNF family known as CD40L, and binding of CD40L to CD40 during cognate T–B interactions provides B cell help (for review, see ref. 8). CD40 is expressed on other APCs (macrophages and dendritic cells) as well (9). Recent studies showing that CD40 engagement induces the expression of CD80 and CD86 (10, 11) suggest an indirect function of this pathway, i.e., to promote CD28-mediated costimulation. However, it also has been reported that engagement of CD40L provides a direct costimulatory signal to the T cell (12). Here, we have addressed this question by examining the ability of anti-CD40L mAb to prevent murine cardiac allograft rejection and modulate the expression of B7 molecules *in vivo*.

MATERIALS AND METHODS

Murine Cardiac Allografts. C57BL/6 (H-2^b) and BALB/c (H-2^d) mice aged 6–8 weeks were purchased from The Jackson Laboratory and housed in pathogen-free conditions. Cardiac allografts from BALB/c donors into C57BL/6 recipients were placed in an intraabdominal location (13). Graft function was assessed daily by palpation. Animals received mAbs or fusion proteins (at a dose of 200 μg unless otherwise stated) by intravenous injection at the time of transplantation or 2 days after engraftment. In some instances, animals also received an intravenous injection of 5×10^6 donor splenocytes at the time of transplantation. Rejection was defined as the day of cessation of palpable heartbeat, and was verified by autopsy and selective pathological examination. Loss of graft function within 48 hr of transplant was considered a technical failure (<10% on average), and these animals were omitted from further analysis.

Monoclonal Antibodies and Fusion Proteins. The anti-CD40L mAb MR-1 and a control hamster Ig were gifts of Randy Noelle (Dartmouth Medical School, Hanover, NH). The anti-CD80 mAb 16-10A1 was a gift of Gary Gray (Repligen, Boston). The anti-CD86 mAb GL1 was obtained from American Type Culture Collection. The fusion protein CTLA4Ig has been described (14). The fusion protein CTLA4IgY100F was produced by introducing a phenylalanine residue at position 100 in place of tyrosine using PCR primer-directed mutagenesis. In brief, *in vitro* and *in vivo* studies indicate that CTLA4IgY100F binds CD80 with similar avidity as does CTLA4Ig, but has at least a 200-fold lower avidity for CD86. *In vitro*, CTLA4Ig has detectable binding (by fluorescence-activated cell sorter) to CD86-transfected Chinese hamster ovary cells at concentrations as low as 10-30 ng/ml, whereas CTLA4IgY100F fails to bind to CD86-transfected Chinese hamster ovary cells at concentrations as high as 100 μg/ml. Both CTLA4Ig and CTLA4IgY100F bind equivalently to CD80-transfected Chinese hamster ovary cells (R.P. and

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Abbreviations: APC, antigen-presenting cell; CD40L, CD40 ligand; DST, donor-specific transfusion; TNF, tumor necrosis factor; IL, interleukin; IFN, interferon.

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P.S.L., unpublished work). The half-life of CTLA4IgY100F in mice is similar to that of CTLA4Ig. CTLA4IgY100F effectively blocks CD80-dependent responses (32).

Immunopathology. Portions of each graft were snap-frozen and stored at −70°C until sectioning, or formalin-fixed and paraffin-embedded for standard light microscopy. Isotype-matched control mAbs and rat and hamster mAbs against mouse proteins were purchased from PharMingen, unless specified. These consisted of mAbs for CD80 (16-10A1) and CD86 (GL1); cell surface markers expressed by all leukocytes (CD45, 30F11.1), T cells (CD5, 53-7.3), B cells (CD45R/B220, RA3-6B2), monocytes (CD11b, M1/70), natural killer cells (NK1.1, PK136), and granulocytes (Gr-1, RB6-8C5); the activation antigens VCAM-1 (CD106, 429) and interleukin (IL)-2R (CD25, 3C7); and the cytokines IL-2 (S4B6), IL-4 (11B11), interferon (IFN) γ (R4-6A2), IL-10 (JES5-2A5), and TNF-α (MP6-XT22), plus a polyclonal antibody to IL-12 (R & D Systems). Cryostat sections were fixed either in paraformaldehyde-lysine-periodate for demonstration of cell surface antigens or in acetone for localization of cytokines, and were stained by a four-layer PAP method as described (15, 16). Each graft was analyzed at three or more levels, with counts of $CD45^+$ leukocytes in 10-20 fields per section (expressed as mean ± SD of cells per high power field). The specificity of labeling was assessed using isotype-matched mAbs or purified Ig. In addition, the specificity of cytokine staining was confirmed by overnight mAb absorption with recombinant cytokines (IL-2, IL-4, IL-10, and IFN-γ, obtained from PharMingen) prior to immunohistologic labelling of selected cytokine-rich day 3 allografts (15, 16).

RESULTS

Effect of anti-CD40L mAb on Cardiac Allograft Survival. We first studied the effect of a blocking hamster anti-murine CD40L mAb on cardiac allograft rejection. As shown in Fig. 1, administration of a single dose of anti-CD40L mAb on the day of transplantation significantly delayed rejection in all recipients, and led to indefinite graft survival in the majority (five of seven recipients). Previously, using a rat model of cardiac allografts, we found that the use of donor-specific transfusion (DST) consisting of donor-type splenocytes, was synergistic with CTLA4Ig in preventing graft rejection (17).

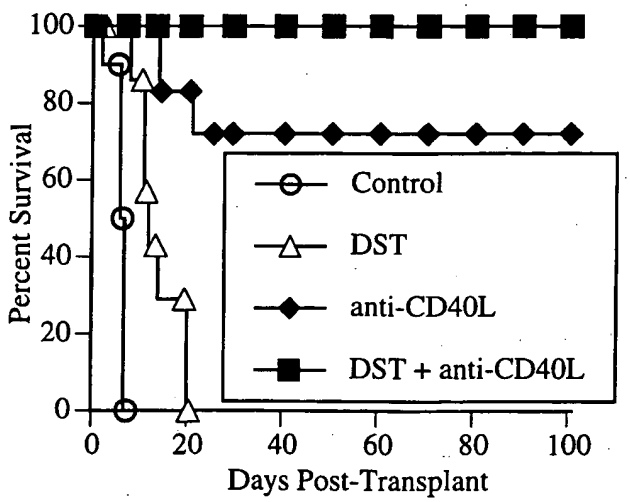

FIG. 1. Effect of anti-CD40L mAb on cardiac allograft survival. BALB/c hearts were transplanted into C57BL/6 recipients. Allograft recipients were treated with either a control Ig or anti-CD40L mAb (200 μg by intravenous injection) at the time of transplantation. Selected recipients also received a transfusion of 5×10^6 donor lymphocytes (DST) at the time of transplantation.

Similar results were reported recently with murine islet allografts, in which a 7-week course of treatment with anti-CD40L mAb (1 week pre- and 6 weeks posttransplant) alone prevented rejection in 40% of animals, whereas the addition of a single DST led to indefinite graft survival in 96% of the recipients (17). To determine if DST similarly improved graft survival in animals treated with anti-CD40L mAb, mice received a transfusion of 5×10^6 donor-type splenocytes at the time of transplantation (Fig. 1). DST alone had a slight graft-prolonging effect, although most grafts were rejected within 2 weeks, and all were lost by 3 weeks. The combination of DST and anti-CD40L mAb was clearly synergistic, with all animals maintaining allograft function beyond 150 days.

Effect of CD40L mAb on Intragraft Cytokine Expression. We next examined the effect of anti-CD40L mAb on the pattern of intragraft cytokine gene expression (Fig. 2 and Table 1). At day 3 after transplantation, control grafts (treated with DST plus control Ig) contained mononuclear cells expressing IL-2 and IFN-γ, but essentially no cells producing IL-4 or IL-10 were detectable. In contrast, while there was an equivalent mononuclear infiltrate in the grafts of DST plus anti-CD40L mAb-treated animals (Table 1), these cells lacked detectable IL-2 or IFN-γ, but showed a striking induction of both IL-4 and IL-10 expression. In additional experiments, we found that the intensity and pattern of expression of IL-2, IL-4, IL-10, and IFN-γ, as well as the extent of the mononuclear cell infiltrate, were identical in animals treated with a control Ig alone compared with animals treated with DST plus control Ig (data not shown). Furthermore, animals treated with anti-CD40L mAb alone (without DST) had identical findings as those treated with anti-CD40L mAb plus DST (data not shown). Thus, the effects of DST plus anti-CD40L mAb on cytokine expression are a specific result of blockade of CD40L. They are neither the result of DST alone nor require the concomitant use of DST. Animals treated with anti-CD40L mAb also had reduced intragraft expression of the proinflammatory cytokines IL-12 and TNF-α (Table 1). Interestingly, anti-CD40L mAb had no effect on the expression of vascular cell adhesion molecule-1, a ligand for Very Late Antigen-4 (Table 1).

Expression of CD80 and CD86 During the *in Vivo* Response to Alloantigen. Given the role of the CD40L-CD40 pathway in modulating the expression of CD80 and CD86 *in vitro* (10, 11), and the known importance of B7 molecules in graft rejection (3, 5), it was important to characterize the normal pattern of CD80 and CD86 expression during an alloimmune response *in vivo*, and to determine the effects of anti-CD40L mAb on these parameters. For these experiments, cardiac allografts were examined by immunohistologic labeling at serial time points posttransplant (Fig. 3). Neither CD80 nor CD86 molecules were detected in significant amounts in the cardiac tissue before transplantation, with only rare and scattered interstitial dendritic cells and an occasional endothelial cell being labeled (Fig. 1, day 0). By 24 hr after transplantation of cardiac allografts, CD86 was densely expressed on virtually all endothelial cells and interstitial dendritic cells. CD86 expression was not increased in control cardiac isografts (Fig. 3) within the limits of detection, suggesting that up-regulation of this molecule is occurring due to specific immune recognition of foreign major histocompatibility complex proteins and the ensuing immune response, and not merely as a result of a nonspecific inflammation secondary to the "trauma" of transplantation (tissue manipulation, ischemia, etc.). In contrast to the prompt up-regulation of CD86 expression, CD80 expression remained unchanged (i.e., weak dendritic cell labeling) within the graft both 24 and 48 hr after transplantation. A striking difference was seen 3 days after transplantation however, at which point CD80 (as well as CD86) was densely expressed on most dendritic cells and infiltrating macrophages, i.e., a population of cells that expressed CD86 at 24 hr. CD80 expression on endothelial cells was more focal and never

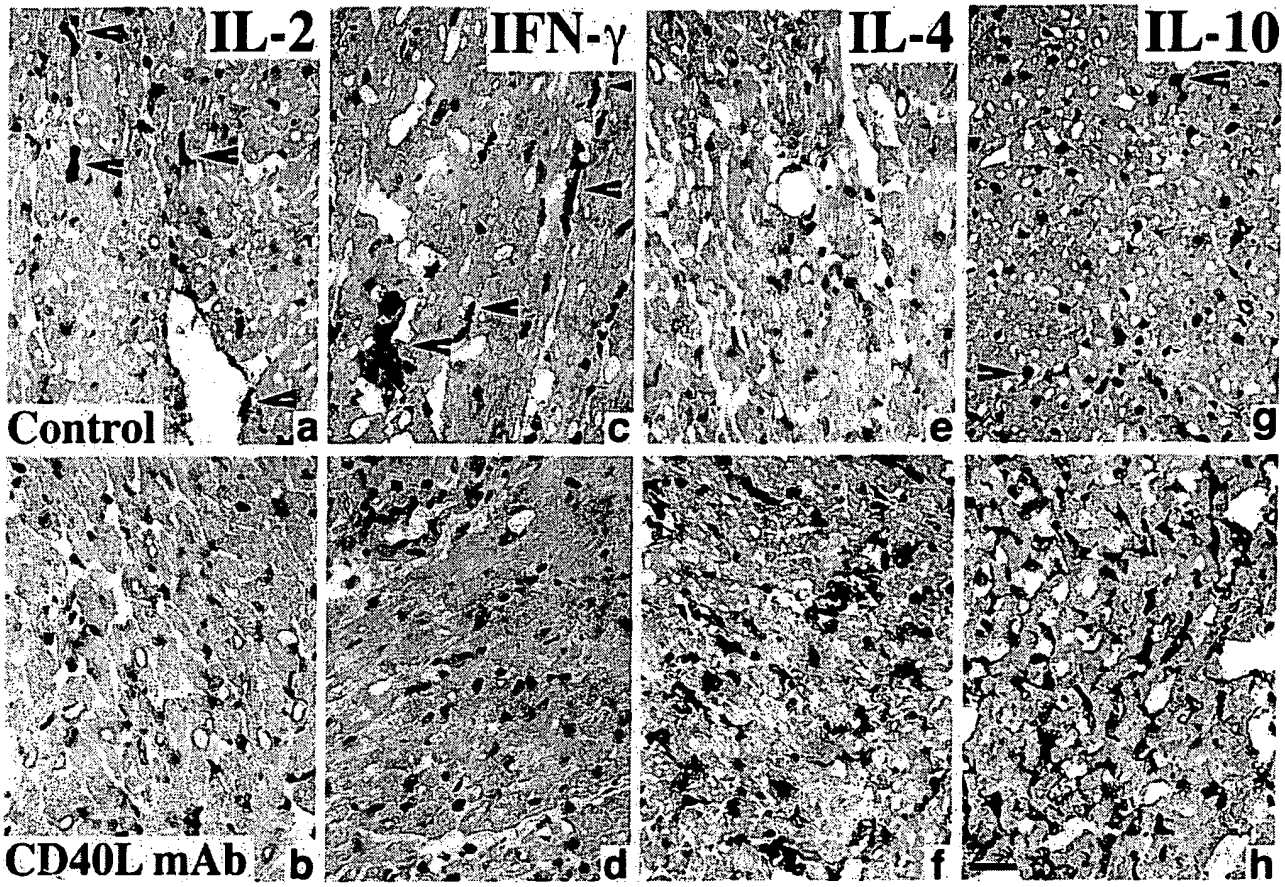

FIG. 2. Effect of anti-CD40L mAb on intragraft cytokine gene expression. C57BL/6 recipients of BALB/c cardiac allografts were treated with either DST plus 200 μg of control Ig (*Upper*) or DST plus 200 μg of anti-CD40L mAb (*Lower*) at the time of transplantation. The animals were killed after 3 days and the hearts were stained for expression of IL-2, IFN-γ, IL-4, and IL-10 as described. Arrowheads (*a* and *c*) indicate the typical mononuclear cell labeling for IL-2 and IFN-γ confined to control grafts, whereas grafts from anti-CD40L mAb-treated recipients showed dense mononuclear and some adjacent endothelial cell labeling for IL-4 (*f*) and IL-10 (*h*). Cryostat sections, hematoxylin counterstain. (Bar = 50 microns.)

appeared to be as strong as CD86 expression on those cells, which remained high even at 3 days.

Effect of CD40L mAb on the Expression of B7 Molecules. To determine whether anti-CD40L mAb affected CD80 or CD86 expression, the animals were killed at day 3 (a time when control animals express both CD80 and CD86 in their grafts; see Fig. 3) for immunopathologic assessment (Fig. 3). By comparison with untreated animals, it can be seen that DST plus control Ig by itself had no observable effect on the pattern or intensity of CD80 or CD86 expression. Interestingly, however, there was a differential effect of anti-CD40L mAb treatment on the expression of CD80 and CD86. While anti-CD40L mAb treatment had no detectable effect on the expression of CD86, it almost completely abrogated the induction of CD80 expression, with treated animals having only a small number of residual $CD80^+$ cells in their grafts, similar to what was seen before transplantation or in control isografts. This was not due to alterations in the populations of cells available for examination, as both control-Ig and anti-CD40L-treated animals had equivalent mononuclear infiltrates as assessed by cell morphology and immunohistochemical staining for T cells, macrophages, natural killer cells, and B cells

Table 1. Immunopathology of day 3 cardiac allografts

Feature	Control Ig plus DST	Anti-CD40L mAb plus DST
Leukocyte infiltration*	Moderate, multifocal infiltrate of T cells (<25%) and monocytes (>75%)	Same
$IL\text{-}2R^+$ cells	10–20% of MNCs	<1% of MNCs
IL-2, IFN-γ	5–10% of MNCs	Negative
IL-4, IL-10	<1% of MNCs	>50% of MNCs and ECs
IL-12, TNF-α	20–50% of MHC and focal ECs	<1% of MNCs
VCAM-1	Most ECs	Same

Comparable data were seen in mice given control Ig alone and mice given DST plus control Ig. Data reflect evaluation of 10-20 fields per graft and three grafts per group. Normal hearts lacked any of these features apart from the presence of small numbers of $CD45^+$ resident dendritic cells and a rare $VCAM\text{-}1^+$ endothelial cell. MNC, mononuclear cell; EC, endothelial cell; VCAM-1, vascular cell adhesion molecule-1.

*Control Ig plus DST grafts contained 43 ± 11 $CD45^+$ leukocytes per high power field, and anti-CD40L mAb plus DST grafts contained 39 ± 12 leukocytes per high power field (P = not significant).

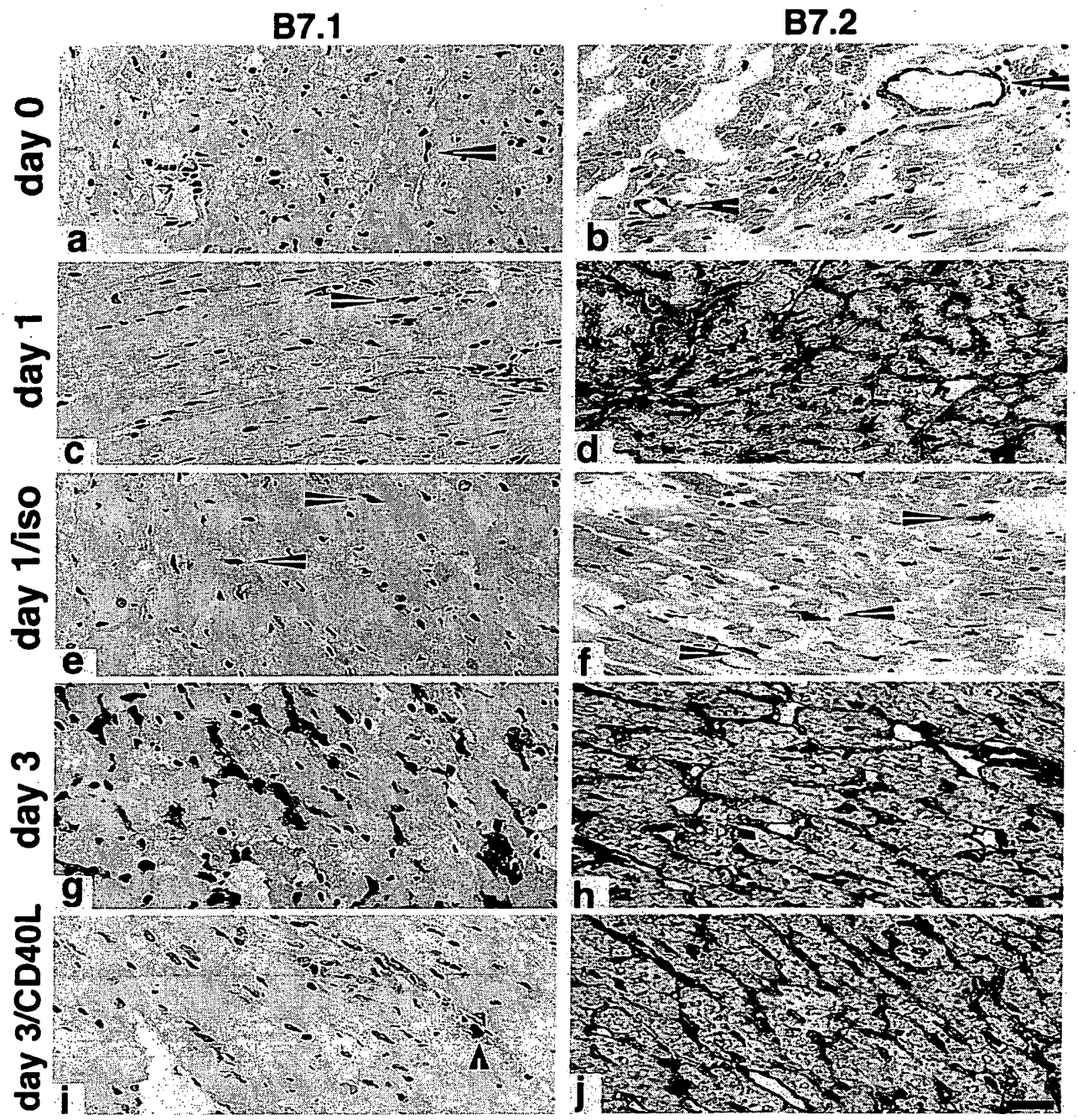

FIG. 3. Serial changes in B7-1 and B7-2 expression within murine cardiac allografts. BALB/c hearts transplanted into C57BL/6 recipients were harvested after 1 or 3 days and sections stained for the expression of CD80 (B7-1) or CD86 (B7-2). Day 0 panels represent a harvested donor heart immediately before transplantation. An isograft control (BALB/c donor into BALB/c recipient) was harvested on day 1. Arrowheads in *a* and *c* indicate the weak labeling for B7-1 that is restricted to interstitial dendritic cells in the first day posttransplantation, whereas by day 3, dense mononuclear and some capillary endothelial cell labeling is seen (*g*). In contrast, B7-2 is expressed by occasional graft endothelial cells (arrowheads in *b*) but is rapidly and densely up-regulated in allografts within 24 hr (*d*) but not in a day 1 isograft (*f*). Where indicated, the recipients were treated with DST plus 200 μg of anti-CD40L mAb at the time of transplantation (*i* and *j*). DST plus CD40L mAb administration blocked up-regulation of B7-1, without affecting graft cellularity (compare *g* and *i*). The dense endothelial expression of B7-2 was not modulated by DST plus anti-CD40L mAb (compare *h* and *j*). DST plus control Ig did not affect the marked increase in intragraft B7-1 seen at day 3 in control allografts (data not shown). Cryostat sections, hematoxylin counterstain. (Bar = 50 microns.)

(Table 1, and data not shown). The lack of effect of anti-CD40L mAb on CD86 expression also serves as a control for cells that are potentially capable of expressing CD80. Therefore, as assessed by immunohistochemical staining of the grafts, we find that the ability of anti-CD40L treatment to prevent acute cardiac allograft rejection, and to promote long-term survival, is associated with selective blockade of CD80 induction. Furthermore, the staining patterns for CD80 and CD86 were not altered by DST alone, and were identical in animals treated with anti-CD40L mAb alone, as in animals receiving anti-CD40L mAb plus DST (data not shown). Thus, as with the findings regarding intragraft cytokine staining (Fig. 2), the effects of anti-CD40L mAb on intragraft staining of CD80 and CD86 are not dependent upon the use of DST.

Table 2. Effects of CD80 and CD86 blockade on graft survival

Treatment	Cardiac allograft survival
Control Ig	6, 7, 7, 7, 7, 7, 8, 8, 8, 8, 8
DST	11, 12, 12, 13, 19, 20, 20
CTLA4Ig + DST	>150 (n = 12)
Anti-B7-1 + DST	20, 22, 23, 42, 42
CTLA4IgY100F + DST	5, 5, 7, 11, 16
Anti-CD86 + DST	7, 25, 28, 100+, 100+
CTLA4IgY100F + anti-CD86 + DST	>150 (n = 4)

BALB/c hearts were transplanted into C57BL/6 recipients. Allograft recipients were treated with a control Ig, anti-CD80 mAb, or anti-CD86 mAb (200 μg by intravenous injection), CTLA4Ig (200 μg), or CTLA4IgY100F (600 μg) 2 days after transplantation. Selected recipients also received a transfusion of 5×10^6 donor lymphocytes (DST) at the time of transplantation.

Effect of Selective CD80- and CD86-Blockade on Allograft Survival. The data in Fig. 3 suggested the hypothesis that the mechanism by which anti-CD40L mAb prevented graft rejection was through inhibiting the induction of CD80 on APCs. Therefore, we next tested the ability of selective blockade of CD80 or CD86 to prevent rejection. As seen in Table 2, and consistent with our previous studies in rats (5, 17), a single dose of CTLA4Ig (given on day 2) plus DST induced indefinite graft survival in all cardiac allograft recipients. In contrast, DST plus anti-CD80 mAb minimally delayed rejection and was unable to induce long-term survival. Blockade of CD86 alone with mAb induced long-term survival in a minority of recipients.

Although the anti-CD80 mAb used in the studies above (16-10A1) clearly blocks CD28 binding (18), it has been suggested that intact anti-CD80 mAbs might have uncharacterized positive signaling effects on CD80-expressing cells (19). Therefore, in additional experiments, we used a mutated form of CTLA4Ig, called CTLA4IgY100F, in which the tyrosine at position 100 was replaced by a phenylalanine. This mutation causes CTLA4IgY100F to retain binding activity for CD80 but abolishes binding to CD86. An advantage of this reagent over Fab fragments of anti-CD80 mAb are its relatively longer half-life (53 hr) compared with expected half-life of Fab fragments *in vivo*. CTLA4IgY100F had no effect, either at the same dose as CTLA4Ig (200 μg, data not shown) or at a 3-fold higher dose (600 μg, Table 2). The inability of CTLA4IgY100F to prevent rejection was not due to failure to adequately block CD80. Table 2 shows that CTLA4IgY100F and anti-CD86 mAb synergize in preventing allograft rejection, indicating that CTLA4IgY100F is an effective competitive inhibitor for CD80 binding. Thus, selective blockade of CD80 *in vivo* is unable to replicate the immunosuppressive effects of anti-CD40L mAb.

DISCUSSION

To our knowledge, this is the first report describing the pattern of expression of the costimulatory ligands CD80 and CD86 *in vivo* during the response to a vascularized organ allograft. We find that both molecules are up-regulated as part of a specific immune response (rather than as a result of the transplant procedure itself), and that CD86 is expressed significantly earlier than CD80. The expression pattern of the two molecules is distinct, in that CD86 appears to be quite a bit more prominent on endothelial cells than does CD80. The endothelium is the first site of contact of host T cells with recipient tissue in the case of vascularized grafts. This, coupled with the known ability of endothelium to express major histocompatibility complex class II molecules, makes it likely that endothelial cells play a very prominent role in the early stages of alloactivation *in vivo*.

Many previous studies attest to the importance of CD80 and CD86 in transplant rejection (5, 17, 20–22). More recent evidence suggests a role for the CD40-CD40L pathway in these responses as well (6–8, 23). This might be as a direct T cell costimulator (12, 24) or via induction of CD80 and CD86 on B cells (10, 11). In addition, CD40 is expressed on macrophages, and ligation of CD40 has been shown to potentiate macrophage production of nitric oxide and selective monokines such as IL-12, and to enhance macrophage cytotoxicity (25).

Our data indicate that blockade of CD40L by administration of a single dose of mAb at the time of transplantation is able to induce long-term survival of vascularized cardiac allografts in ≈70% of murine recipients. The addition of DST leads to indefinite graft survival in all animals, although the mechanisms by which DST augment the effects of anti-CD40L mAb are not known. Parker *et al.* (23) have shown that anti-CD40L antibody combined with DST blocked rejection of murine islet allografts, although in that system prolonged administration (2–7 weeks) of the antibody was required. Our model, one of vascularized organ transplantation, introduces an additional level of complexity.

Recently, Larsen *et al.* (26) have shown that anti-CD40L mAb alone could prevent murine cardiac allograft rejection in the majority of recipients. In their studies, CTLA4Ig, given at the time of transplantation, did not prevent ultimate graft rejection, consistent with our own previous reports regarding the need to delay administration of CTLA4Ig (17). However, combination of anti-CD40L mAb and CTLA4Ig initiated at the time of transplantation was synergistic, leading to long-term survival in all animals (27). While anti-CD40L mAb alone did not effect the expression of T cell cytokines or of B7 molecules, the combination of CTLA4Ig plus anti-CD40L inhibited the expression of IL-2, IL-4, IL-10, and IFN-γ, as determined by reverse transcription–PCR. Transcripts for CD80 and CD86 were only minimally affected. This is in contrast to our own data, where blockade of CD40L alone induced a Th2 immune deviation in association with a loss of CD80 expression. The reasons for this discrepancy are not immediately apparent, but may relate to their use of multiple doses of anti-CD40L mAb to prevent rejection, or may be due to the use of different detection methods [protein detection by immunohistochemistry in the present study versus mRNA detection by RT-PCR in Larsen *et al.* (26)].

It is important to note that our results of costimulatory molecule and cytokine expression were all obtained using immunohistochemistry. An advantage of this technique is the ability to detect protein itself, and the preservation of tissue architecture, allowing for spatial localization of the relevant gene products. While we cannot exclude low residual CD86 expression or a small affect on CD80 expression in the grafts of anti-CD40L-treated animals, the large alterations in CD80, CD86, and cytokine gene expression seen in the present study are real, and seem likely to be physiologically meaningful.

Endothelial cells, when activated, are immunogenic, expressing major histocompatibility complex class I and II molecules, adhesion receptors, and costimulatory molecules. Recently, three groups have shown that CD40 is expressed on endothelial cells and that ligation of CD40 on endothelial cells *in vitro* up-regulates intercellular adhesion molecule-1, E-selectin, and vascular cell adhesion molecule-1 (28–30). In that system, neither CD80 nor CD86 was induced by CD40 ligation of endothelium (28). While that signal may not by itself induce these molecules, our data clearly indicate that, in the case of alloimmune responses, CD40-mediated signals are required for CD80 induction *in vivo*.

In is also interesting to note that treatment with anti-CD40L mAb was able to prevent rejection in the face of abundant CD86 expression throughout the graft endothelium and graft infiltrating APCs. This result implies that long-term graft survival can be achieved without blockade of either T-cell antigen receptor-mediated signals or the CD28 pathway. Thus,

while CD28-mediated costimulation may be necessary for graft rejection, our data suggest that it is not sufficient (at least not when CD86 is the ligand), and that signals mediated through CD40L-CD40 interactions are required as well. Although extremely unlikely, it should be noted that we cannot exclude a direct effect of anti-CD40L mAb on TCR or CD28 signaling.

In our studies, prolongation of graft survival by anti-CD40L mAb was accompanied by immune deviation toward Th2 cytokines, and by specific down-regulation of CD80 expression without an effect on CD86 expression. Recently, Stüber *et al.* (31) reported that anti-CD40L Ab prevented Th1-mediated inflammatory colitis by blocking the secretion of IL-12, an effect observed in our study as well (Table 1). Whether or not the loss of Th1 and induction of Th2 cytokines we observed is responsible for the graft prolonging effects of anti-CD40L mAb is not known. This pattern of selective cytokine sparing is associated with enhanced graft survival in a variety of models; however, a causal role for Th2 cells in transplantation tolerance has yet to be established.

Our studies define the effects of CD40L-blockade on the expression of the T cell costimulators known to be required for transplant rejection (i.e., CD80 and CD86), and suggest that the effects of anti-CD40L mAb on these molecules alone cannot account for its tolerogenic effects in organ transplantation. We cannot, however, completely exclude the possibility that anti-CD80 mAb and CTLA4IgY100F provided only incomplete blockade of CD80, relative to the inhibition seen with anti-CD40L mAb. Further experiments using CD80-knockout mice will be required to answers this question as well as to address the potentially distinct roles of CD80 on donor compared with recipient cells, and on T cells versus APCs.

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(10) RELATED PROCEEDINGS APPENDIX
NONE.

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